Protein crosslinking

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by

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"He said science was going to discover
the basic secret of life someday," the bartender put in.
He scratched his head and frowned.
"Didn't I read in the paper the other day
where they'd finally found out what it was?"
"I missed that," I murmured.
"I saw that," said Sandra. "About two days ago."
"What is the secret of life?" I asked.
"I forget," said Sandra.
"Protein," the bartender declared.
"They found out something about protein."
"Yeah," said Sandra, "that's it"

Kurt Vonnegut
"Cats Cradle" (1963)

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ABSTRACT

This thesis is primarily concerned with the DHA-mediated formation of non-disulfide protein crosslinks as a result of the Maillard reaction. Prior to investigating this reaction, an efficient method for the preparation of pure DHA hydrate was optimised and the compound characterised.

DHA was shown to react with amino acids and amino acid derivatives and a separation technique was developed which enabled identification of two reaction products. DHA was shown to react with model proteins, and the formation of DHA-mediated covalent crosslinks between protein monomers was demonstrated using an electrophoretic technique, which had previously been optimised. The effects of various conditions on the crosslinking reaction were examined. Mechanistic studies provided compelling evidence that crosslink formation was occurring at the ε-amino group of the lysine residue. Various degradation products of DHA, including cyclotene, the structure of which was definitively determined by X-ray crystallography, were shown to crosslink model proteins under standard conditions. Analysis of the products of the cyclotene-protein reaction system, by electrospray mass spectrometry, indicated that the successive addition of four cyclotene molecules to the protein had occurred, providing evidence for a Schiff base intermediate in the crosslinking reaction.

The latter stages of this thesis explored an enzyme-mediated mechanism for the introduction of non-disulfide protein crosslinks. The enzyme TGAse was shown to crosslink HMW glutenin subunits *in vitro*. Proteins extracted from a bread dough containing TGAse were analysed by SE-HPLC and compared to those from a control dough. TGAse was found to decrease the SDS-soluble glutenin fraction and increase the SDS-insoluble glutenin fraction. The water soluble proteins and gliadins remained unchanged. TGAse was shown to have a profound effect on the properties of a baked loaf. Loaves to which TGAse had been added demonstrated an increase in the volume of water which can be maintained by the dough, increased crumb strength, improved texture and a decrease in the optimum work input. These previously unreported effects show great promise for its use as a processing aid in the bulk manufacture of white pan bread.

ABBREVIATIONS

0°C std	zero degree standard
2,4-DNP	2,4-dinitrophenylhydrazine
2-DAA	2-deoxy-2-(N-ε-(N-α-acetyllysin)-yl)-ascorbic acid
3-DAA	3-deoxy-3-(N-ε-(N-α-acetyllysin)-yl)-ascorbic acid
3DG	3-deoxyglucosone
3-DT	3-deoxythreosone
4-VP	4-vinylpyridine
A_{340}	absorbance reading at 340 nm
A_{460}	absorbance reading at 460 nm
AA	ascorbic acid
AMPS	10% ammonium persulfate solution
B. Pt.	boiling point
br	broad
Bro	potassium bromate
*t*Bu	tertiary butyl group
°C	degrees centigrade
C_n	n^{th} carbon
^{13}C NMR	carbon nuclear magnetic resonance
CBB	Coomassie brilliant blue
CML	N-ε-(carboxymethyl)lysine
d	doublet
Δ	heat
Da	Daltons
δ_C	carbon NMR chemical shift
dd	doublet of doublets
δ_H	proton NMR chemical shift
DHA	dehydroascorbic acid

dH_2O	distilled water
DKG	2,3-diketogulonic acid
DMSO	dimethyl sulfoxide
DNP	2,4-dinitrophenylhydrazine positive spot (thin layer chromatography)
DPI	N-α-acetyl-N-δ-(4-(1,2-dihydroxy-3-propyliden)-3-imidazolin-5-on-2-yl)-L-ornithine
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ESR	electron spin resonance
FTP	formyl threosyl pyrrole
g	gram
^1H NMR	proton nuclear magnetic resonance
HMF	5-hydroxymethylfurfural
HPLC	high performance liquid chromatography
HMW glutenins	high molecular weight glutenin subunits
inc. std	incubated standard
IR	infrared
kDa	kiloDaltons
kg	kilogram
L	litre
LL	3-(N-ε-lysino)-lactic acid
m	multiplet
mA	milliAmp
MDD	mechanical dough development
Me	methyl group
mg	milligram
MHz	megaHertz
MS	mass spectrometry
m/z	mass:charge ratio
µg	microgram
µL	microlitre
mL	millilitre
M. Pt.	melting point
p.p.m.	parts per million
N	Newton

NAD^+	nicotinamide adenine dinucleotide - oxidised form
NADH	nicotinamide adenine dinucleotide - reduced form
NMR	nuclear magnetic resonance
NPS	ninhydrin positive spot
ODA	oxalic acid di-N-ε-(N-α-acetyl)lysinamide
OMA	oxalic acid mono-N-ε-(N-α-acetyl)lysinamide
PAGE	polyacrylamide gel electrophoresis
q	quartet
qu	quintet
R_f	retention factor
RNAse A	ribonuclease A
RP-HPLC	reversed-phase high performance liquid chromatography
r.p.m.	revolutions per minute
s	singlet
SCA	scorbamic acid
SDS	sodium dodecyl sulfate
SE-HPLC	size exclusion-high performance liquid chromatography
t	triplet
TEMED	N,N,N',N'-tetramethylethylenediamine
TGAse	transglutaminase
TLC	thin layer chromatography
Tris	Tris(hydroxymethyl)aminomethane - free base
UTM	universal testing machine
UV	ultraviolet
v/v	unit volume per unit volume
w	weak
Wh/kg	Watt hours per kilogram
w/v	unit weight per unit volume

INTRODUCTION

1.1 Background

Proteins are one of the most important and chemically complex categories of organic compounds, arguably forming the basis of life. They are a class of diverse macromolecules composed of long chain polymers of L-α-amino acids. Each of the twenty naturally occurring amino acids found in proteins has a distinctive R-group ranging from a single hydrogen atom, as in glycine, to the heterocyclic ring structure of tryptophan (see *figure 1.1*).

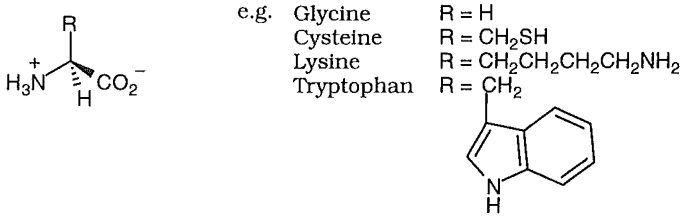


Figure 1.1: L-α-amino acid structure.

Proteins carry out many of the most important functions required for maintaining and replicating cells in organisms. These range from the catalysis of biochemical reactions by enzymes, to muscle contraction, the transport of ions across cell membranes, and the maintenance of structure by fibrous proteins such as collagen.[1] Proteins are also important in foods, where they play important nutritional and functional roles.[2]

1.2 Structural organisation of a protein

Up to four levels of organisation can exist in a protein. Primary structure is determined by the linear sequence of amino acids. The primary structure, therefore, constitutes a complete description of the covalent connections of a protein, as synthesised on the ribosome. The alteration of a single amino acid may affect an essential characteristic of a protein.

Secondary structure refers to the spatial arrangement of amino acid residues that are near one another in the linear sequence. Some of these steric relationships are of a regular kind, α-helices and β-pleated sheets for example. Such local, regular folding gives rise to a periodic structure.[3] The helically coiled regions are, in turn, folded to give a specific compact structure. This is referred to as the tertiary structure of the protein and describes the overall spatial arrangement of the amino acid residues. At this level of organisation a number of forces help determine the structure and, hence, the function of the protein. These forces include electrostatic bonds, Van der Waals interactions, hydrophobic interactions, hydrogen bonds and covalent crosslinks. It is with these covalent crosslinks that this thesis is concerned.

Proteins containing more than one polypeptide chain have an additional level of organisation called the quaternary structure, which refers to the relative orientation and interaction between chains. Quaternary structure may also be influenced by covalent crosslinks.

1.3 The relationship of structure to function

The precise ordering of amino acids in protein chains is ultimately responsible for the overall three dimensional structure and shape of the protein. In turn, the structure determines the protein's properties and, therefore, its function. Enzymes, for example, are proteins which contain a small area known as the active site. The structure of this area complements the structure of the substrate and

facilitates binding. Factors that affect the three dimensional structure of an enzyme, particularly the shape of the active site, may therefore affect its biological activity. For instance, alterations in pH can interfere with the electrostatic interactions that may contribute to the maintenance of the enzyme's conformation.[4]

Other extrinsic factors, such as the temperature and ionic environment of the protein, combined with its intrinsic characteristics, may affect the conformation of the protein molecule, thereby contributing to the size and shape of the protein. This is particularly relevant for food proteins, where metabolic regulation may no longer be functioning. The presence or absence of covalent crosslinks can also significantly alter the properties of a protein; this has been implicated during food processing,[5] cataract formation,[6] arthritis,[7] and Alzheimer's disease.[8]

Hen egg white lysozyme and pancreatic ribonuclease A are two examples of proteins stabilised by intramolecular crosslinks. Each protein contains four disulfide bridges in its native conformation.[9,10] These crosslinks are believed to confer an element of thermal stability to the protein.[11] For instance, lysozyme is quite stable to heat, requiring a denaturation temperature of 75°C, at pH 7.0, to unfold the protein.[12] The heat stability and high denaturation temperature are responsible for many of the properties of egg white observed during cooking. That is, covalent crosslinks may alter the functional properties of food proteins. The influence of crosslinks on the functionality of food proteins has been reviewed;[13,14] however, the mechanisms involved in their formation are not fully understood.

1.4 Why study protein crosslinking?

As discussed above, naturally occurring covalent crosslinks can have a profound effect on the structure and function of a protein. The appropriate degree of crosslinkage is critical for maintaining the correct degree of firmness or elasticity for a particular cell, tissue or

organ in any biological system.[14] In addition to their fundamental interest, methods of modifying the properties of a protein by changing its crosslinking pattern are, potentially, of great practical importance. Two areas where these methods are of particular value are in medicine[15] and in the food industry.[16]

1.4.1 Importance of crosslinking reactions in medicine

Crosslinking plays an important role in many biological processes, in particular blood clotting, as well as providing an important method of introducing strength into mechanical proteins such as collagen and elastin. It has been implicated in diabetes, Alzheimer's disease and the ageing process.

Diabetes

Protein crosslinking has been implicated in some of the physiological complications faced by diabetic patients.[17] In non-diabetics, the hormone insulin is produced when a rise in blood glucose is detected. Diabetics, however, have a functional insufficiency of insulin which impairs the ability of the body to utilise glucose. Many diabetic patients, therefore, have to inject insulin once or twice a day in order to control the concentration of glucose in the blood. This offers a poor mimic of the normal feedback system and results in large fluctuations of glucose in the bloodstream. Ageing processes such as the development of cataracts, kidney disease and artherosclerosis, are known to occur at an earlier age in diabetics than in non-diabetics.[18] This is presumably due to increased Maillard chemistry, described in section 1.6.2, which represents a series of reactions involving carbonyl-containing compounds and free amine groups, such as that of the amino acid lysine. These reactions may lead to an increase in the number of protein crosslinks.

The ageing process

Glucose-induced crosslinkage occurs to some degree in non-diabetics, where blood glucose is at a normal level. These crosslinks accumulate over time and are, therefore, of great importance in the ageing process. Collagen, for example, gradually accumulates an increasing number of crosslinks, making the protein increasingly less elastic and more brittle. As a result, skin becomes less elastic and tendons become less supple. Covalent crosslinks also contribute to the arterial rigidity commonly associated with ageing.[19]

Alzheimer's disease

Maillard chemistry has been implicated in the development of Alzheimer's disease, a form of dementia, which is characterised by the formation of neurofibrillary tangles and plaques.[20] These insoluble protein deposits have been found to accumulate in the brains of Alzheimer patients.[8] The inherent stability of these protein aggregates, ensuring a long half life, makes them ideal substrates for glycation. Exactly how they are formed, however, remains unclear.

1.4.2 Importance of crosslinking reactions of food proteins

The crosslinking of food proteins can have a profound effect on the properties of the proteins which, in turn, can effect the quality of the food product. The deliberate modification of food proteins, either enzymatically or chemically, may prove to be a valuable tool for the manipulation of food properties.

The blocking of deteriorative reactions

Food proteins must undergo a number of potentially damaging processes, such as harvesting, processing and storage, before being consumed.[21] Each of these processes could adversely affect the structure of the protein by changing its crosslinking pattern. Such changes can be brought about by chemical reactions, by enzymes that

are already present in the foodstuff, or by reagents introduced during processing.

Much effort has been devoted to the study of these changes, which are generally perceived to be detrimental to product quality, in the hope of finding methods to minimise their impact.[22-24] For example, the Maillard reaction may reduce the content of intact essential amino acids, particularly the amino acid lysine, and slow down or halt the release of these amino acids during digestion. It may also lead to the inhibition of proteolytic enzymes, as well as to the production of various mutagenic and carcinogenic compounds, further reducing the nutritional value of the food.[25]

There have been attempts to protect proteins against the Maillard reaction by the chemical modification of free amino groups. Modifications such as acetylation, methylation, isopropylation and cyclopentylation,[26] have prevented the interaction of sugars with lysine residues. The nutritional availability of the modified lysine, however, is not known.

The improvement of physical properties

Some functional properties which may be directly affected by the presence or absence of covalent crosslinks in food proteins are: texture, solubility, emulsification, viscosity and gelling properties. The solubility and emulsifying activity of food proteins have been shown to improve with the addition of covalent crosslinks, introduced *via* the enzyme transglutaminase (TGAse).[27] The same enzyme has been used to crosslink meat proteins, improving the texture of restructured meat products.[28] Food proteins, crosslinked by the Maillard reaction, have also demonstrated improved gelling properties, with breakstrengths much higher than those of monomeric protein.[16]

The baking industry has been chemically modifying dough proteins for many years, resulting in improved consistency, texture and

strength of many baked goods.[29] The addition of oxidising agents has been found to improve the performance of doughs. The mechanism of this improvement is thought to be due to the formation of disulfide bonds.[30]

1.5 Enzyme-mediated crosslinkage

1.5.1 Disulfide crosslinkage

Disulfide bonds are the best characterised type of protein crosslink and are formed from the oxidative coupling of two cysteine residues, which are close in space, within a protein. This form of crosslinking requires the presence of a suitable oxidant and may or may not be enzyme-catalysed. The oxidant accepts the hydrogen atoms from the thiol groups of the cysteine residues producing the disulfide bridge. The position of the equilibrium is determined by the redox potential of the environment.

Several enzymes are reported to regulate thiol-disulfide interchange and thereby modify the functional properties of proteins. Such enzymes include: sulfhydryl oxidase;[31] peroxidase;[32] protein disulfide isomerase;[33] protein disulfide reductase;[13] and lysyl oxidase.[34]

One of the better known proteins containing intermolecular disulfide crosslinks is insulin. Insulin is a hormone produced in the pancreas which helps regulate the breakdown of sugar. Its primary sequence was determined by Sanger and co-workers in 1955.[35] Insulin contains one intramolecular disulfide bond and two intermolecular disulfide bonds. They are initially formed as intramolecular crosslinks in a single polypeptide chain. Proteolytic cleavage of the intervening section between the two end sections of the polypeptide results in the formation of two chains held together by the two intermolecular disulfide bonds.[1]

1.5.2 Non-disulfide crosslinkage

There are a number of characterised non-disulfide covalent crosslinks in proteins. Collagen is one of the most abundant proteins in animals, generally found in the connective tissue. Tendons, for example, are almost entirely composed of collagen. The immense strength required of this protein is provided, in part, by the crosslinks between its three intertwined peptide strands.[36]

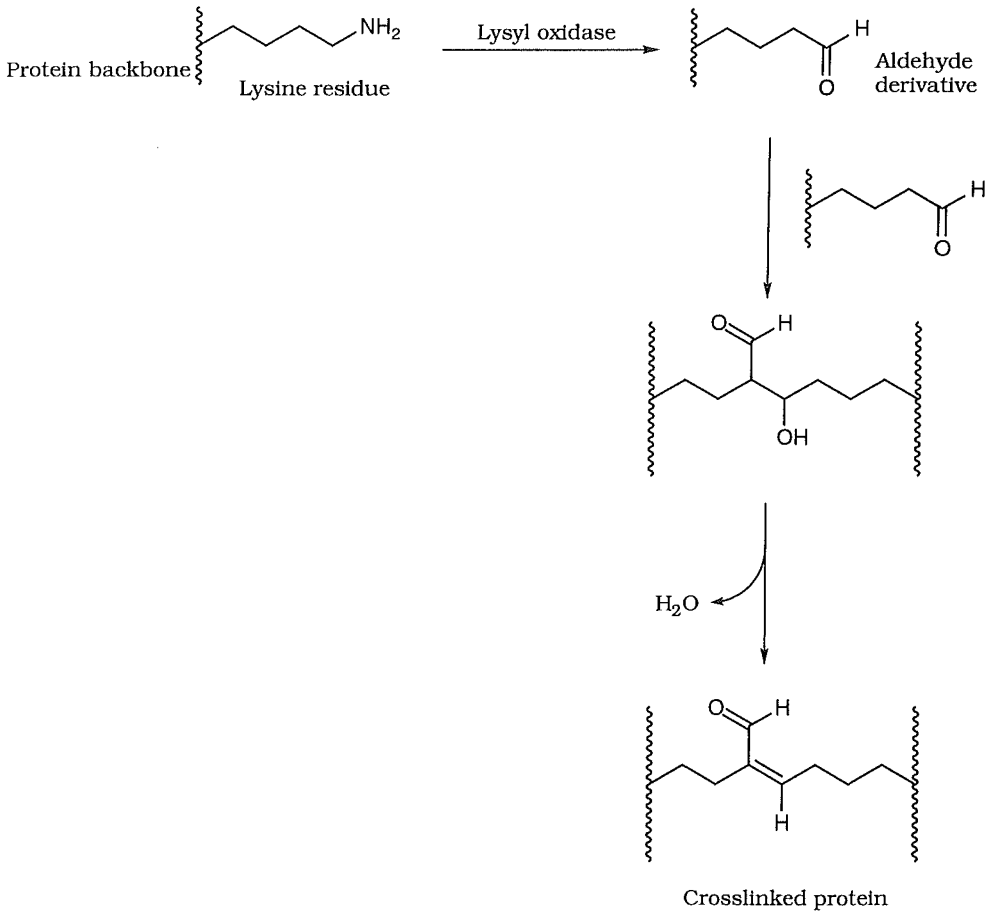


Figure 1.2: aldol crosslinking of the protein collagen.

The enzyme lysyl oxidase is critical in the initial steps of the synthesis of the crosslinks in collagen.[36] They are formed *via* the oxidation of specific residues of lysine and hydroxylysine to aldehyde

derivatives. These then react spontaneously with the side chain of a second oxidised lysine residue *via* the aldol reaction (see *figure 1.2*).

The protein elastin is found in tissues such as ligaments and arterial blood vessels. Whereas collagen is a relatively rigid protein, elastin consists of highly elastic fibres. As such, the crosslinks in elastin are chemically very different to those of collagen, although their formation also involves the enzyme lysyl oxidase. The side chain of one lysine residue from each of four polypeptide strands combines to form a desmosine crosslink[34] (see *figure 1.3*).

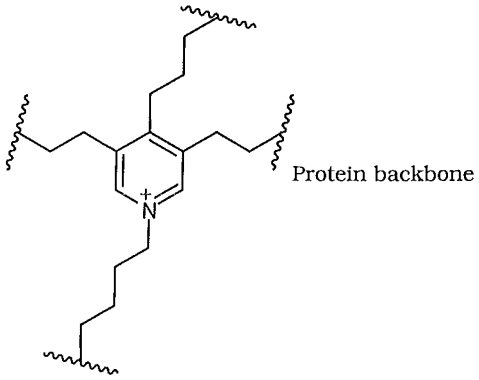


Figure 1.3: the desmosine crosslink found in the elastin fibre.

A third example of non-disulfide crosslinking can be found in the mechanism of blood clotting, where a cascade of reactions leads to the conversion of fibrinogen, a water-soluble protein, to fibrin, a water-insoluble one, which can then aggregate to form a soft blood clot.[37] This is then stabilised by a reaction, catalysed by TGAse, also known as factor XIII, which results in the formation of a network of covalent crosslinks, thereby generating a hard clot (see *figure 1.4*).

TGAse has been purified and used to crosslink various proteins of interest, particularly food proteins, including casein,[38] and proteins

from soy,[39] meat[28] and wheat.[40] Until very recently, studies have been limited by a lack of readily available enzyme.[41]

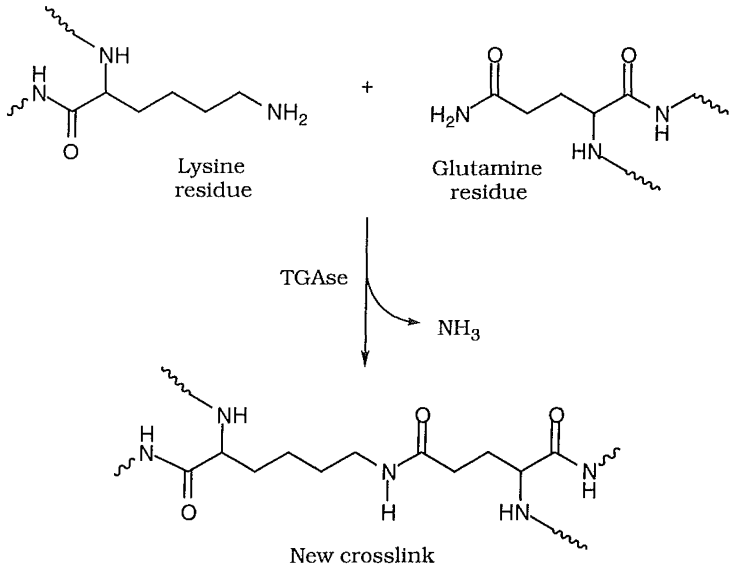


Figure 1.4: blood clot formation via crosslinkage catalysed by the enzyme TGAse.

1.6 Crosslinkage via chemical modification

1.6.1 Disulfide crosslinkage

In biological systems, the redox chemistry resulting in disulfide formation is often carried out by the ascorbic acid-dehydroascorbic acid (DHA) redox couple shown in *figure 1.5*.[42] Ascorbic acid, commonly known as vitamin C, is an effective reducing agent. It has been shown that reactive thiol groups of major haemoglobins from guinea pig, rat and cat can reduce DHA to ascorbic acid with the resulting formation of intrachain disulfide bonds, without enzyme catalysis.[43]

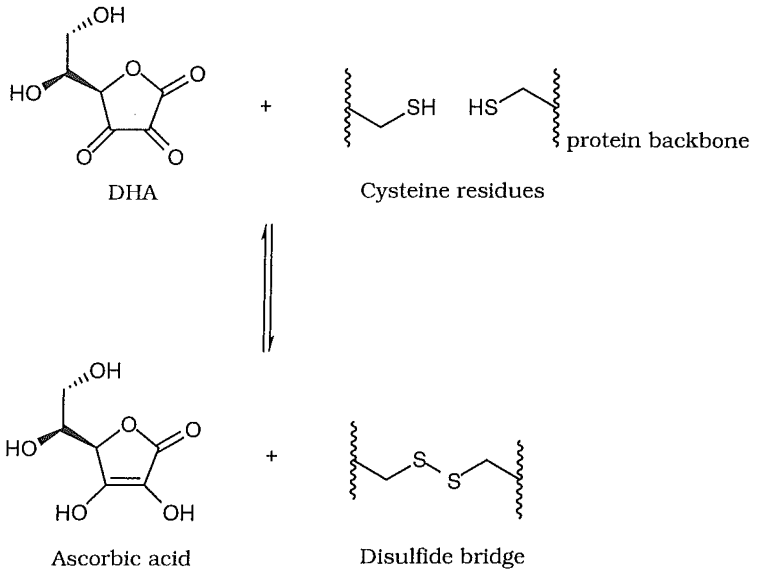


Figure 1.5: the redox reaction resulting in the formation of a disulfide bridge.

1.6.2 Non-disulfide crosslinkage

Non-disulfide crosslinkages can be formed *via* various chemical reactions and can be artificially introduced into a protein using a wide range of chemical reagents. Such crosslinking reagents are often bifunctional molecules with chemically reactive groups, chosen for their specificity for a particular chemical group; others are less specific.[44,45] Chemical crosslinking reagents are of importance in industry and pharmacology, as well as in fundamental research.[46,47]

Recent studies have highlighted the existence of naturally occurring non-disulfide crosslinks which are often a result of Maillard chemistry.[23,48]

Maillard chemistry

The Maillard reaction, first described by L.-C. Maillard in 1912,[49] is a simplistic description of a complex series of reactions involving the reaction of free amino groups, such as amines, amino acids, peptides

and proteins, with carbonyl compounds, particularly reducing sugars.[50] The reaction, sometimes referred to as non-enzymatic browning, glycation or glycosylation, begins with the formation of a Schiff base between the carbonyl group of the sugar and the free amino group. This then rearranges to give a more stable ketosamine, known as an Amadori product, as shown in *figure 1.6*.[25]

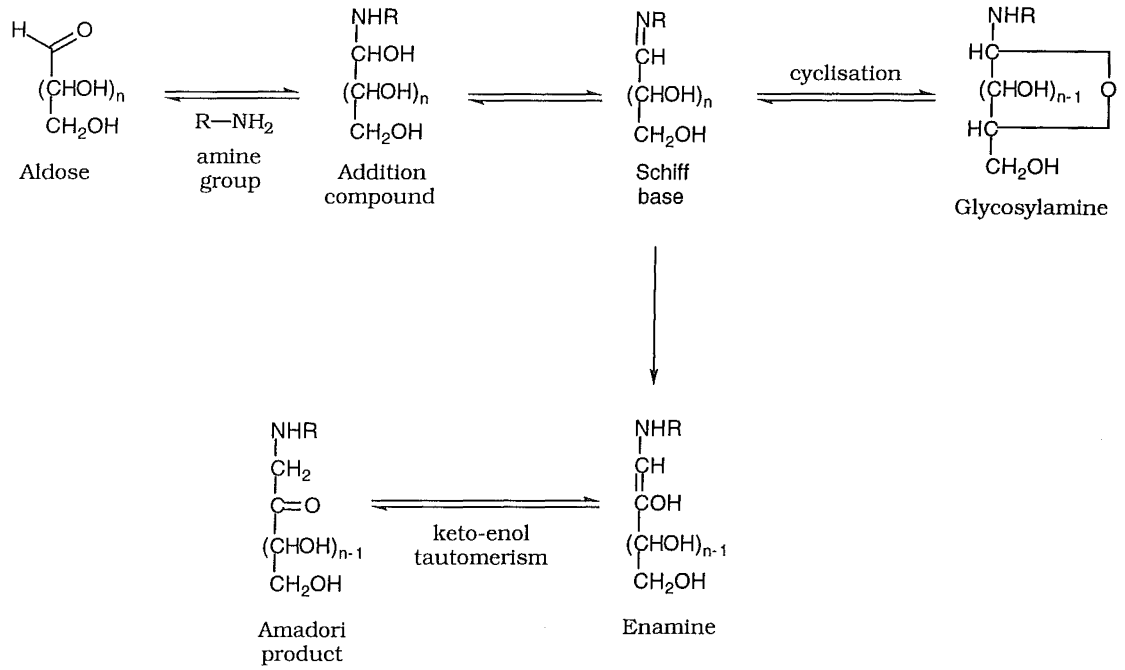


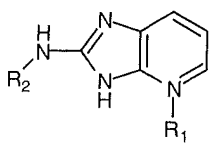
Figure 1.6: initial reactions of Maillard chemistry.

a) Maillard reaction - Low molecular weight products

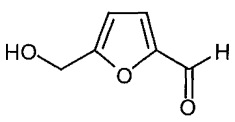
The Amadori product can undergo any of a variety of reactions including acid hydrolysis, oxidation, enolisation, cyclisation, dehydration[25,51] and free radical reactions.[52,53] These reactions lead to the formation of a complex mixture of compounds. Reaction conditions, such as reaction time, concentration of reactants, temperature and pH,[25] help determine which of the vast array of compounds are produced, and in what concentrations. The Maillard reaction is particularly important in food systems where the products of the reaction can be responsible for the aroma, taste and appearance of foods. They can also cause deterioration of food during

food storage and processing, causing a decrease in nutritional quality through the formation of antinutritional and toxic compounds, the destruction of essential amino acids and reduced digestibility of food proteins.[50] The Maillard reaction is also increasingly acknowledged as important in medicine.[25]

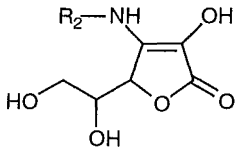
Despite over eighty years of research on the Maillard reaction, its products have only recently started to be accurately identified. These include the compounds pentosidine,[54] formyl threosyl pyrrole (FTP),[6] N-ε-(carboxymethyl)lysine (CML),[55] 5-hydroxymethylfurfural (HMF),[56] 3-deoxyglucosone (3-DG)[57] and 3-deoxy-3-(alkylamino)ascorbic acid (3-DAA)[58] (see *figure 1.7*). The mechanistic pathways leading to the formation of products such as these are gradually being elucidated.[51]



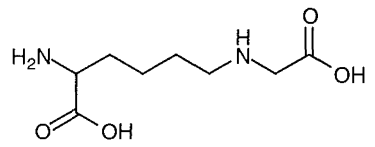
Pentosidene
R_1 = lysine
R_2 = arginine



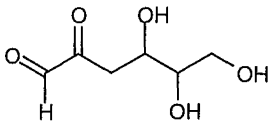
5-Hydroxymethyl
-furfural (HMF)



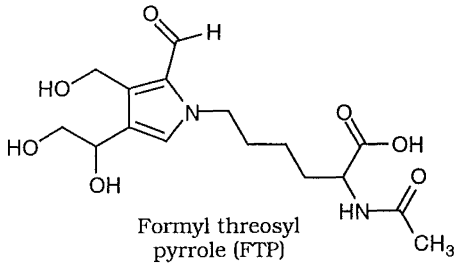
3-Deoxy-3-(alkylamino)
-ascorbic acid (3-DAA)
R_2 = alkyl group



N-ε-(Carboxymethyl)
-lysine (CML)



3-Deoxyglucosone
(3-DG)



Formyl threosyl
pyrrole (FTP)

Figure 1.7: some of the few identified products of the Maillard reaction.

b) Maillard reaction - Protein aggregates

As well as being responsible for the production of low molecular weight compounds, evidence suggests that the Maillard reaction is involved in the formation of protein aggregates *via* covalent crosslinking.[16] Many low molecular weight compounds, such as those shown in *figure 1.8* could result in the formation of crosslinks.[51] However, since the Maillard reaction is very complex, and the chemistry of macromolecules is intrinsically difficult to study, little is known about the molecular details associated with this process.

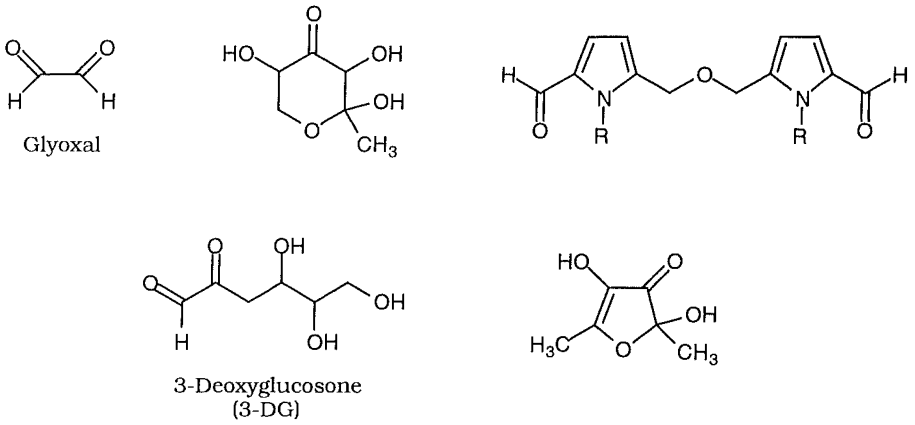


Figure 1.8: low molecular weight compounds which may be involved in protein crosslinkage.

A possible mechanism of crosslink formation is shown in *figure 1.9*.[59]

Recent studies in ophthalmology[60] and aging[15] have highlighted the importance of protein crosslinking by the Maillard reaction in biological systems. One of the proteins in the body with a long half-life is the lens protein of the eye known as crystallin. This protein becomes increasingly modified over time and is believed to lead to age-onset cataract.[61] Protein crosslinks may also play a role in diabetic cataract formation, as diabetics often have higher than normal blood sugar levels.[62] Both disulfide and non-disulfide crosslinks have been implicated in glycation model systems,[63] but recent studies have shown that treatment of crosslinked crystallin

with the reducing agent 2-mercaptoethanol had no affect on the amount of crosslinked protein, suggesting that disulfide bonds are not the predominant crosslinks in this case.[64] The precise mechanism of formation of these crosslinks has not yet been ascertained.

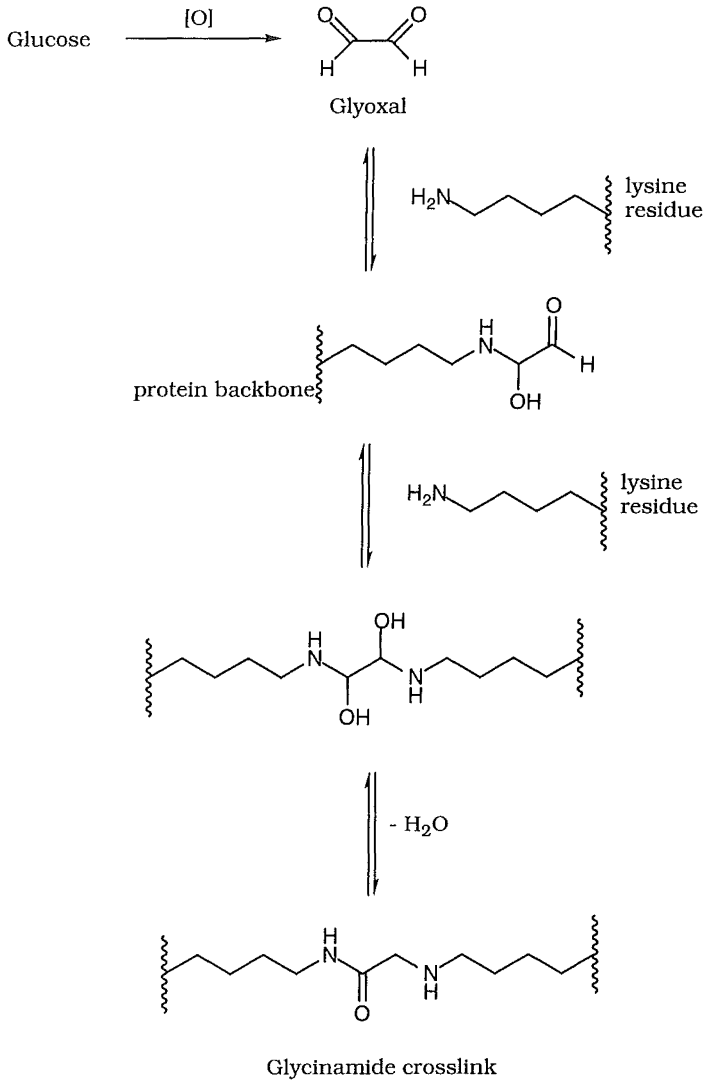


Figure 1.9: the proposed formation of a glycinamide crosslink by glyoxal, an oxidation product of glucose.

The Maillard reaction is of particular interest to those in the food industry since processing conditions can influence the course of the reaction. Research in the food area has concentrated on the identification and formation of low molecular weight compounds

during food processing. Little information is available about the reactions leading to the formation of protein aggregates *via* crosslinkage. More research is required so that the fundamental chemistry of this mechanism can be unveiled. It will then be possible to manipulate the reaction for positive effect, by the deliberate introduction or removal of covalent crosslinks. A thorough understanding of the mechanisms involved may, therefore, provide a tool for the improvement and maintenance of food quality.

c) Improvement of properties related to acceptability, such as flavour and colour

Consumers tend to select food products on the basis of sensory perception. Qualities such as aroma and colour, therefore, play an important role in the production of food products.

The Maillard reaction is the main route to the colour, flavour and aroma properties of foodstuffs.[51,65] Although the development of colour is the most obvious effect of the Maillard reaction, there have been relatively few investigations into the mechanism of colour development. Instead, the majority of recent research has been devoted to the identification of aroma and flavour compounds,[66-68] which are believed to result from the reaction of sugars with amino acids, rather than with the intact protein.[69]

d) The improvement of nutritional properties

The nutritive value of a protein depends not only on the proportions of its essential amino acids but also on their biological availability. The formation of chemically and enzymatically resistant crosslinks in the protein matrix tends to reduce the digestibility of proteins. The steric hindrance of digestive enzymes results in the decreased nutritional availability of the protein's constituent amino acids.[70]

Unmodified lysine is, in principle, biologically available. When a sugar has reacted with the lysine residue to produce a Schiff base, the

reversibility of the reaction results in its continued availability. However, once an Amadori compound has been produced, it appears that the lysine is no longer biologically available.[51] Crosslinking by the enzyme TGAse does not reduce the lysine content, and may in fact protect the lysine residues from deteriorative reactions that often occur during food processing. TGAse catalyses the formation of an amide bond, at the free amino group of the lysine residue, which prevents Maillard chemistry but does not reduce the nutritional availability of the amino acid.[71]

Since the amino acid lysine is nutritionally limiting in many foods, particularly grain-based foods, investigation into the possible fortification of food products with lysine is of some importance. The incorporation of lysine into wheat gluten, by the enzyme TGAse, has been demonstrated by Ikura *et al.*[72] The nutritional quality of the gluten protein is therefore enhanced since both the incorporated amino acid and the protein-bound lysine residue appear to be nutritionally available.[73]

1.7 Overview of this thesis

Protein crosslinking is attracting increasing attention in the scientific literature, in a wide variety of subject areas. A detailed study of the mechanisms of this process, and its practical implications, is therefore very timely.

This thesis is concerned with the formation of covalent crosslinks in proteins. It will initially investigate DHA-mediated crosslinkage in an effort to uncover some of the mysteries underlying the chemical mechanism by which covalent crosslinks can be introduced. This will be followed by an examination of enzymatically modified protein, in particular, the functional effects of the crosslinks introduced by the enzyme TGAse.

1.7.1 Dehydroascorbic acid-mediated crosslinkage

Although important in many biological systems, monomeric DHA is remarkably ill-characterised. *Chapter two*, therefore, will begin with a description of an optimised method for preparing DHA, its subsequent purification and characterisation. Later chapters will delve into the reactions of DHA, utilising both amino acid and protein model systems.

1.7.2 Enzyme-mediated crosslinkage

The latter sections of this thesis will investigate the effects of the deliberate TGAse-mediated introduction of covalent crosslinks into wheat proteins. This will be followed by an analysis of the crosslinking in an actual food system to which TGAse has been added.

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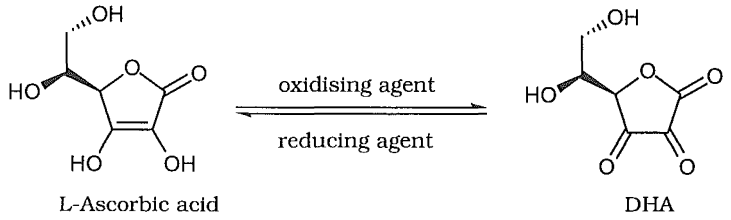
THE CHEMISTRY OF DEHYDROASCORBIC ACID

2.1 Background

L-Ascorbic acid is a common food additive and one of the few 'chemicals' that consumers are happy to ingest in large quantities. It is often added to food to improve the nutritive qualities, for example to supplement the amount of ascorbic acid which is naturally present, or to restore that which was lost during food processing. Ascorbic acid can also function as an antioxidant. In this case, it is present as a preservative and prevents unwanted oxidation of food components. In addition, it has been used as a flour improver for many years; addition of ascorbic acid to a dough improves its elasticity and gas retaining properties and results in a larger loaf with improved texture.[1]

The action of ascorbic acid as a flour improver has generally been attributed to the first stable oxidation product of ascorbic acid, dehydro-L-ascorbic acid (DHA). As can be seen in *figure 2.1*, the two compounds exist in a redox equilibrium, and have equivalent biological utility.[2] Since interconversion between the two compounds occurs readily under physiological conditions, addition of either DHA or ascorbic acid may often result in the same effect.



L-Ascorbic acid DHA

Figure 2.1: L-ascorbic acid-DHA redox equilibrium.

Most flour improvers used in the baking of bread, including potassium bromate, are oxidising agents. Since ascorbic acid is a reducing agent, it has been generally assumed that it is oxidised to DHA which, in turn, acts as an oxidising agent and forms disulfide crosslinks in the gluten matrix.[3] During the mixing of a dough, atmospheric oxygen oxidises the ascorbic acid. That DHA can form disulfide bonds from thiols non-enzymatically has been demonstrated unequivocally[4] and DHA-mediated crosslinkage of haemoglobin has been shown to be due to disulfide bonding.[5] However, this does not preclude alternative reactions.

The redox chemistry of DHA has been widely studied in cereal chemistry, and, with very few exceptions,[6] has overshadowed other possible reactions. In other areas, ascorbic acid is known to react with proteins in Maillard type chemistry,[7] presumably *via* oxidation to DHA with subsequent reaction of the C2 carbonyl group.

A literature search revealed that this possibility had not been studied in detail, with the exception of work on eye lens proteins. These studies suggest that Maillard chemistry is, indeed, responsible for DHA-mediated crosslinking *in vivo*.[8] This chemistry may, therefore, also be occurring during food processing and may be at least partly responsible for the functional effects of ascorbic acid.

Analogous chemistry is known to occur with the sugar glucose both *in vivo*[9] and in food systems.[10] The Maillard chemistry of glucose is relatively well characterised, since it is found in high concentrations in the body and in some food systems, as described in *chapter one*. It

has provided the basis for a number of investigations into protein aggregation *via* covalent crosslinking.[11,12] Various other sugars have also been extensively examined, including ribose,[13,14] xylose[15,16] and fructose.[17,18]

Unlike ascorbic acid, which is a well characterised compound, DHA is remarkably ill-characterised, and its reaction chemistry is poorly understood despite its equally common occurence. It is the aim of the first part of this thesis, therefore, to shed some light on both the chemistry of DHA and its role in the Maillard chemistry of proteins. In particular, to investigate the crosslinking potential of DHA *via* Maillard chemistry, as opposed to its involvement in disulfide bond formation. This thesis will also seek to highlight recent work pertinent to this area, particularly since the last review of DHA chemistry in 1982.[19]

2.2 Preparation of dehydroascorbic acid

Before exploring the reactions of DHA with amino acids and proteins, it was important to have an effective method for the preparation of pure DHA, and to fully characterise the compound and its degradation products. Although DHA is commercially available, our NMR analysis, shown in *figure 2.2*, demonstrated the presence of degradation products of DHA, due to over-oxidation. Therefore, methods of preparing pure DHA were investigated.

DHA can be prepared from ascorbic acid using any of a variety of oxidising agents, for example the halogens,[20] quinones[21] and oxygen.[22] An important chemical property of ascorbic acid is the relative ease with which it undergoes oxidation. Therefore, one of the problems associated with the preparation of DHA is not the actual oxidation of ascorbic acid, but the prevention of over-oxidation. We found ^{13}C NMR to be a convenient means of monitoring the reaction. Careful monitoring of standard literature methods revealed many preparations produced impure product, mainly due to either insufficient oxidation or excessive oxidation.

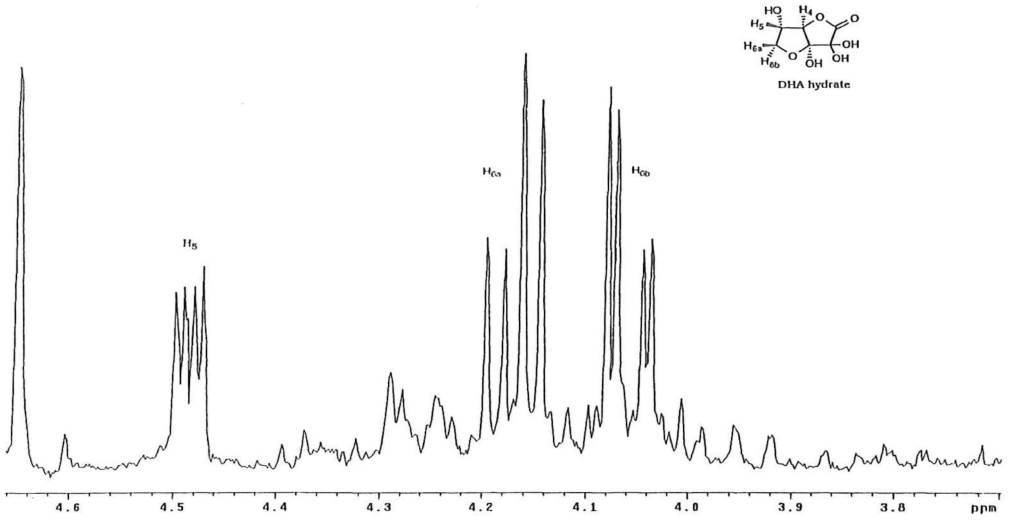


Figure 2.2: ^{1}H NMR of a commercial preparation of DHA.

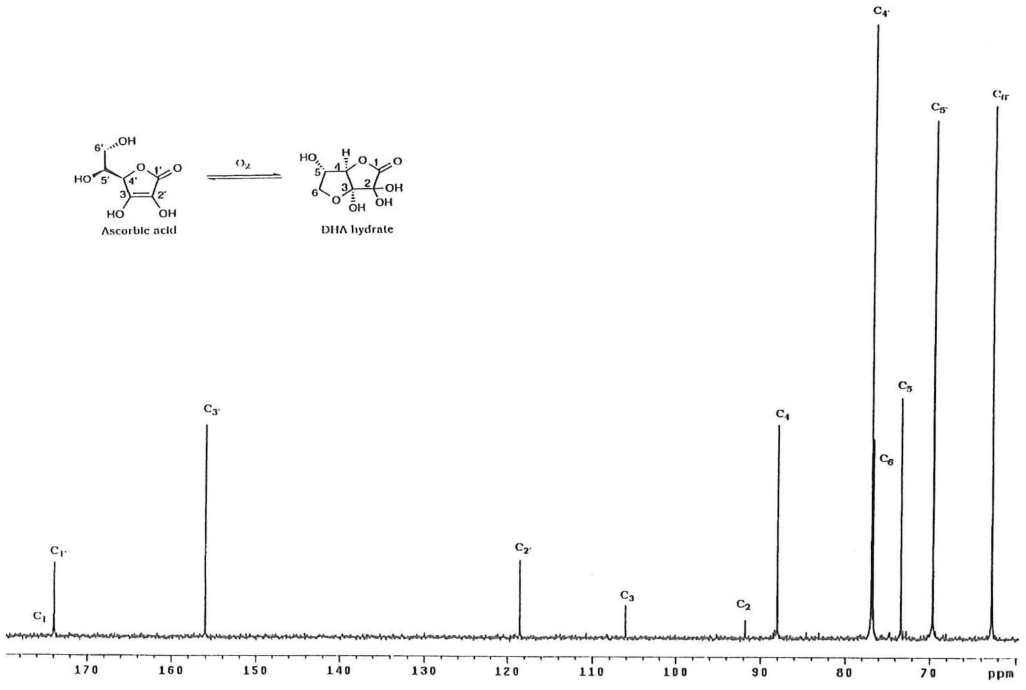


Figure 2.3: ^{13}C NMR of partially reacted ascorbic acid.

As can be seen in *figure 2.3*, this literature procedure gave a twelve line spectrum, indicating a mixture of both unreacted ascorbic acid and DHA, all of which were consistent with literature values.[19]

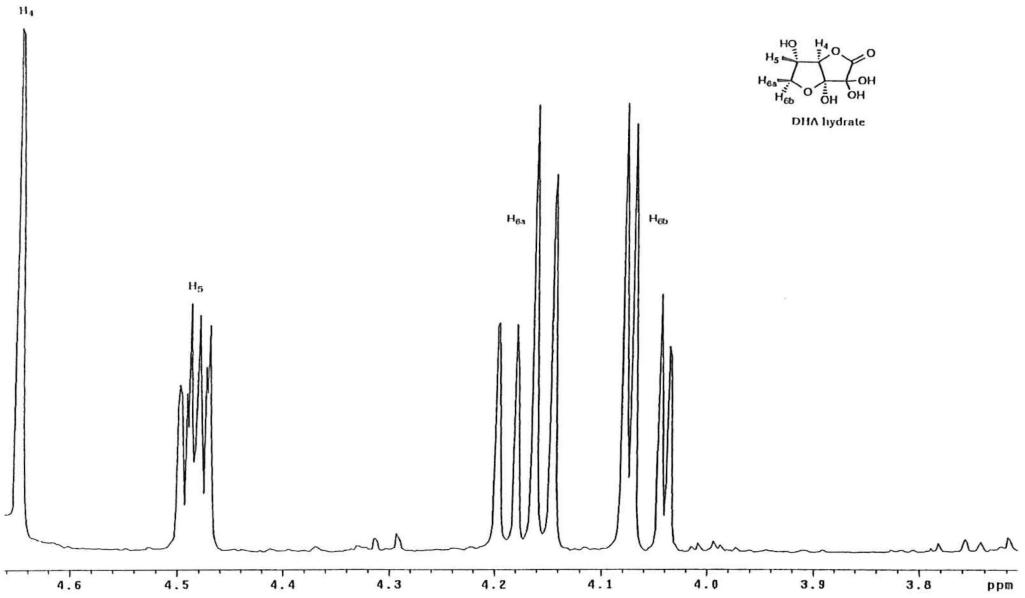
2.2.1 Optimised preparation of monomeric dehydroascorbic acid

The preparation found to be of the most value in quantitatively producing pure, monomeric DHA hydrate (hereafter referred to as DHA) was that developed by Ohmori *et al.*, using oxygen as the oxidising agent.[22] This involves the dissolution of ascorbic acid in a suitable solvent, followed by the addition of activated charcoal as a catalyst. Oxygen was bubbled through the gently stirred solution until oxidation was judged complete by ^{13}C NMR. After the reaction, the activated charcoal was removed by filtration and removal of the solvent was achieved by extensive rotary evaporation. The resulting syrup was repeatedly lyophilised, to remove residual solvent. The product was then stored at -10°C.

A variety of solvents were investigated. Preparation of DHA using the solvent methanol is believed to result in the formation of up to 10-20% of a methanol-DHA complex,[19,20] so was not used for the routine preparation of DHA. Acetic acid also provided relatively clean DHA, but complete removal of the acid could not be achieved, therefore, it was also not routinely used. Ethanol gave the cleanest product with the highest yield (94%) and was selected for the routine preparation of DHA.

The conversion of ascorbic acid to DHA is supported by the diagnostic resonances of carbons two and three, as can be seen in *figure 2.3*. A decrease from 118.5 p.p.m. and 156.0 p.p.m. respectively for ascorbic acid, where they are in an enediol form, to shifts of 91.7 p.p.m. and 106.1 p.p.m. for DHA, where they are in the hydrate, and cyclic hemiketal forms, respectively.

a) ^{1}H NMR spectrum of pure DHA.



b) ^{13}C NMR spectrum of pure DHA.

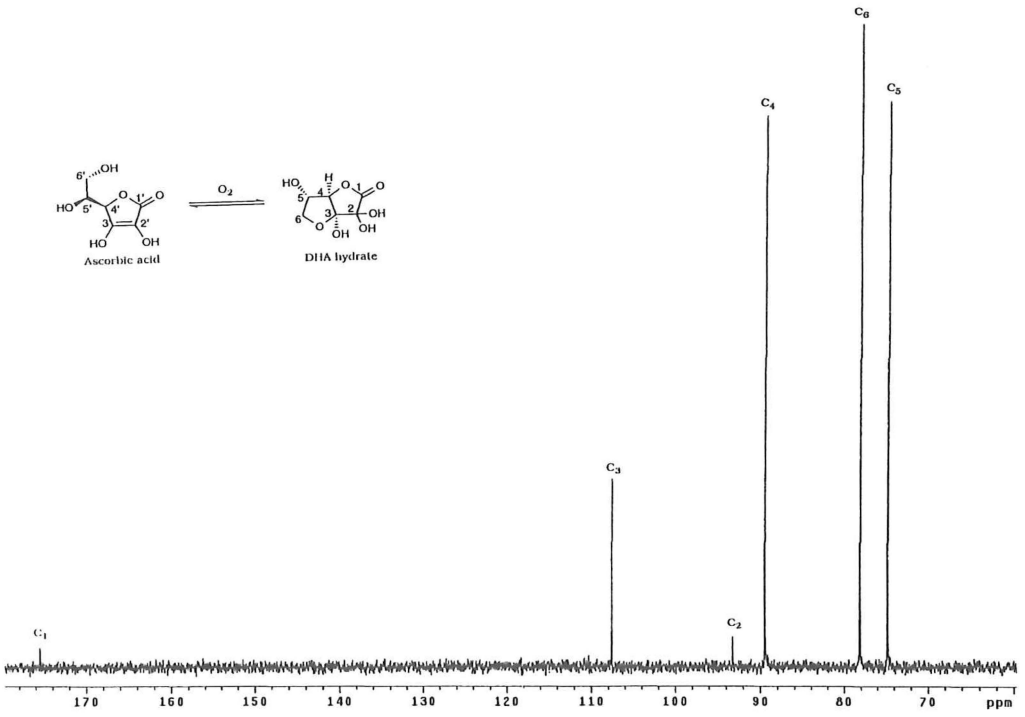


Figure 2.4: ^{1}H and ^{13}C NMR spectra of pure DHA.

When reaction time was increased, compared with that of the literature method, the ^{13}C NMR spectrum showed that complete oxidation to DHA had occurred (*figure 2.4*). The ^{1}H NMR confirmed the purity of the product.

2.3 *Characterisation and properties of dehydroascorbic acid*

2.3.1 *Structure of dehydroascorbic acid*

The solution structure of DHA is well documented.[2] In aqueous solution, DHA exists predominantly in a bicyclic hemiketal form, shown in *figure 2.5*. This is thought to be in equilibrium with small quantities of several other forms.[19]

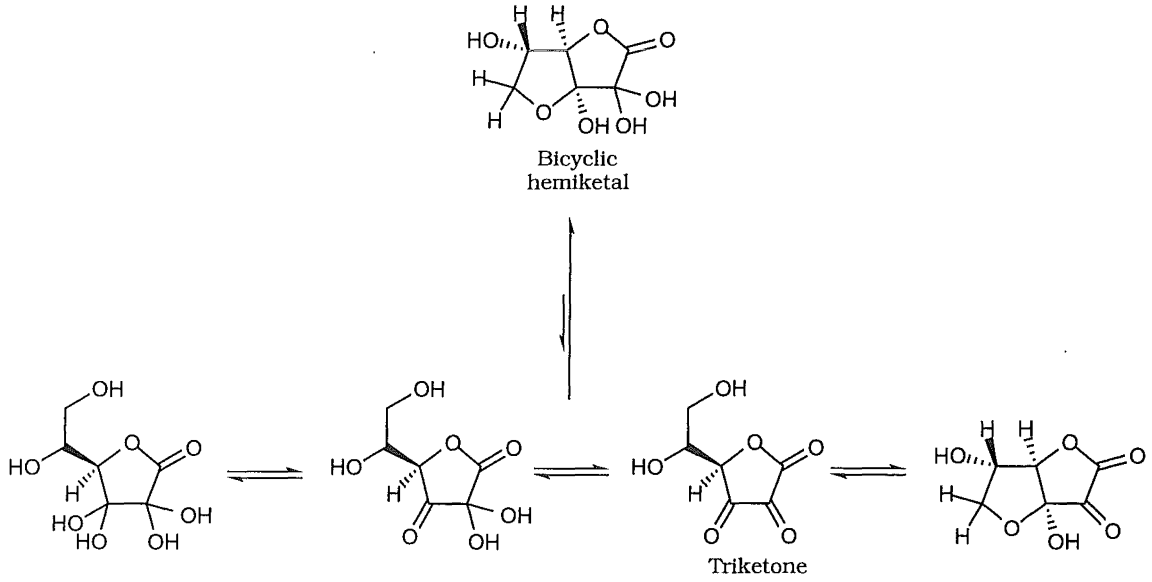


Figure 2.5: predominant and minor forms of DHA in aqueous solution.

We propose that whilst the triketone is only present in small concentrations, its high reactivity makes it the most important form of DHA, in relation to Maillard chemistry. Strong electrostatic interactions exist within this molecule, as all three keto groups are present in the plane of the molecule.[24] Hence, it is very unstable and

nucleophilic attack at the C2 position, by amino groups for instance, is favoured.

2.3.2 Crystalline dehydroascorbic acid

The preparation of dimeric dehydroascorbic acid

We prepared dimeric DHA directly from ascorbic acid using benzoquinone as the oxidising agent. Over-oxidation is known to occur using this procedure, resulting in large quantities of decomposition products in the sample.[25] It was, therefore, not used for the routine preparation of DHA.

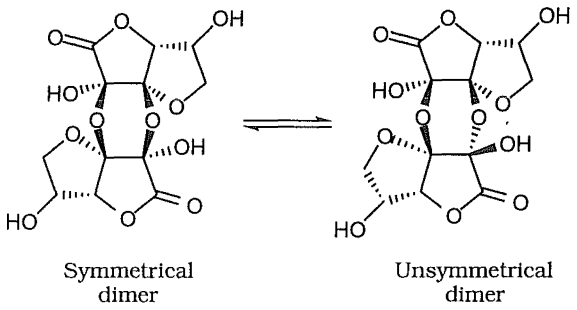


Figure 2.6: structures of dimeric DHA.

Dimeric DHA is readily obtained in crystalline form and its structure has been fully characterised by X-ray crystallography.[26] Solution studies of dimeric DHA by Hvoslef *et al.*,[27] suggest that in non-aqueous solvents at room temperature, an equilibrium is established between a symmetric dimer and an unsymmetric dimer (*figure 2.6*). When as little as 0.4% water is added to a 0.1 M DHA solution, in either dimethyl formamide or dimethyl sulfoxide, all dimers rearrange to give the hydrated monomers shown in *figure 2.5*. Unlike monomeric DHA, dimeric DHA is only sparingly soluble in aqueous solutions.[20]

Monomeric DHA has not previously been reported in crystalline form, rather it is generally obtained as a thick syrup or as an amorphous, microcrystalline solid.[20] In an effort to promote crystal growth, we

prepared samples of pure DHA syrup, by the modified method of Ohmori *et al.*, described above, and dissolved them in a variety of solvents, at a range of temperatures, over various lengths of time. A sample of DHA, held at -10°C for a period of ten months, successfully furnished crystals. NMR confirmed the identity of the material as DHA. When mounted on the X-ray diffractometer, these crystals provided rotation photographs consistent with single crystalline material (*figure 2.7*).

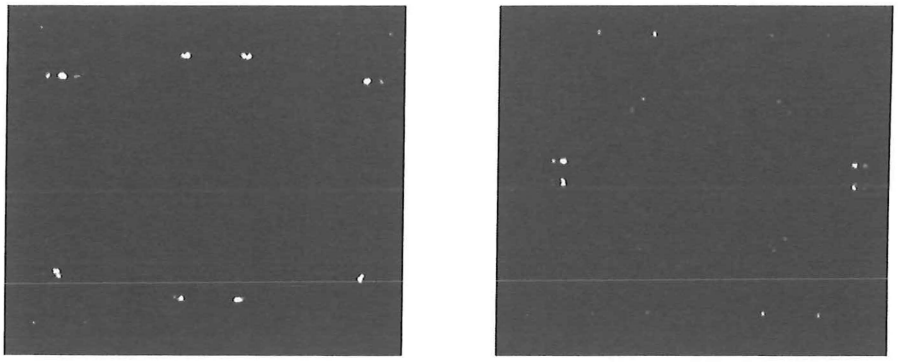


Figure 2.7: rotation photographs of a crystal obtained from a DHA syrup stored at -10°C for ten months.

Unfortunately, the quality of the crystals and their diffracting power were not sufficient for data collection and structure solution. Crystallography of monomeric DHA is particularly troublesome due to the extreme difficulty in obtaining crystals, combined with their very low melting point. Attempts are currently being made to obtain better quality crystals with the use of a laser crystallisation device. This device is specifically designed to grow single crystals of compounds which are liquid or gaseous at ambient temperatures.[28]

2.4 *Reactions of dehydroascorbic acid*

Various reactions of DHA have been investigated by a number of researchers and are briefly reviewed below. The reactions of DHA with amino acids are omitted and will be discussed in detail in *chapter three*.

2.4.1 Reactions of dehydroascorbic acid with hydrazines and amines

Two reaction systems which have been extensively studied are the reactions of DHA with either *o*-phenylenediamine or arylhydrazines. The compounds resulting from these reactions are of value in the synthesis of various heterocycles.[29]

Reaction with arylhydrazines

Regioselective hydrazonation at C2 of DHA has been demonstrated by El Ashry *et al.*[30] This reaction resulted in the formation of the hydrazone of the monocyclic form of DHA, as shown in *figure 2.8.* The lower reactivity of the C3 carbonyl moiety was attributed to its involvement in the formation of the hemiketal ring.[31]

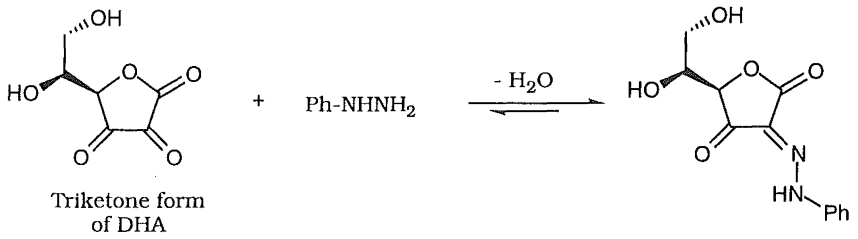


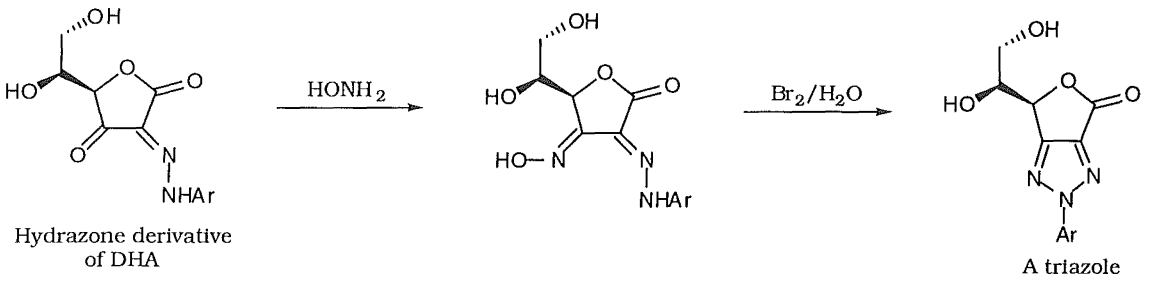
Figure 2.8: structure of hydrazone derivatives of DHA.

This is consistent with our supposition that the triketo form of DHA is the chemically important species during reactions with amines, and that the C2 carbonyl is a likely candidate for Maillard chemistry.

The hydrazone derivatives of DHA can be further reacted with amine compounds such as hydroxylamine, which can, in turn, cyclize to give triazoles (*figure 2.9)* or react with a variety of aldehydes to produce imidazolines.[32]

Reaction of DHA with two molar equivalents of arylhydrazine resulted in the crystallisation of a red coloured bishydrazone whose structure was stabilised by intramolecular hydrogen bonding. Subsequent

rearrangement resulted in the scission of the lactone bond and the formation of a pyrazolone ring (*figure 2.10*).[31]

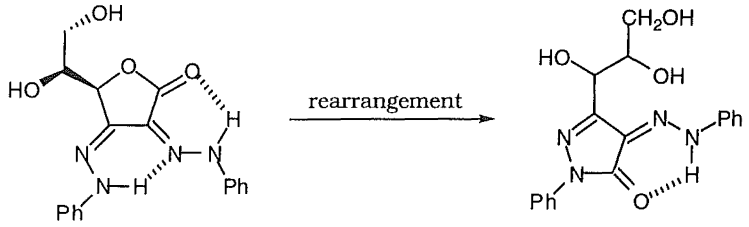


Hydrazone derivative of DHA

A triazole

Figure 2.9: formation of a triazole from hydrazone derivatives of DHA.

Successive reaction of the C2 and C3 carbonyls of DHA has, therefore, been demonstrated and suggests that analogous reactions with protein amino groups, may lead to the formation of crosslinks.



Bishydrazone

Pyrazolone derivative

Figure 2.10: rearrangement of the bishydrazone of DHA to a pyrazolone ring.

Reaction with o-phenylenediamine

The labile nature of Schiff base adducts of DHA has been illustrated with *o*-phenylenediamine.[33] The Schiff base initially formed, can rearrange to give products which depend on the stoichiometry of the reaction, as shown in *figure 2.11*.[33]

The reaction of DHA with one molar equivalent of *o*-phenylenediamine, in a 25% mixture of methanol and water, is

proposed to proceed by the initial nucleophilic attack at C2. Subsequent loss of a water molecule and, in the absence of competing nucleophiles, intramolecular nucleophilic attack at C3, the next most reactive keto group, occurs. When DHA is reacted with two molar equivalents of *o*-phenylenediamine, further nucleophilic attack at the C1 carbonyl group occurs. This type of reaction may also occur in protein systems where nucleophilic attack by an amino group such as that of the lysine residue, could result in crosslink formation.

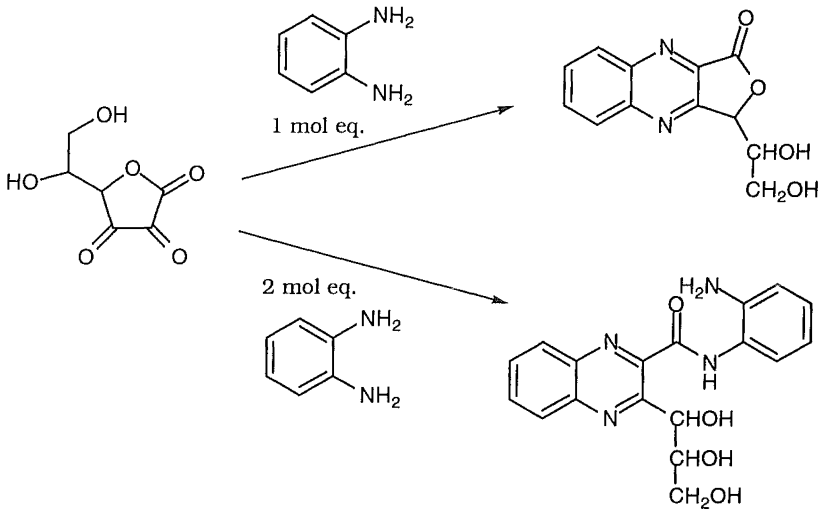


Figure 2.11: products of the reaction of DHA with o-phenylenediamine

2.4.2 Derivatisation of dehydroascorbic acid

In order to extract further information about the structure and reactions of DHA, the preparations of a number of derivatives were attempted. As shown in *figure 2.12*, DHA was reacted with 2,4-dinitrophenylhydrazine,[34,35] methanol,[36] iron (III), copper (II) and silver (I) salts in attempts to prepare structurally characterisable derivatives. Unfortunately, the products of these reactions did not yield further structural information, since numerous products appeared to form in each reaction. Similarly, ascorbic acid was successfully derivatised as an acetonide[37] and various exploratory attempts to prepare a protected derivative of DHA were investigated.

However, the oxidation of the acetonide yielded only DHA, the protecting group presumably having been lost by acid hydrolysis.

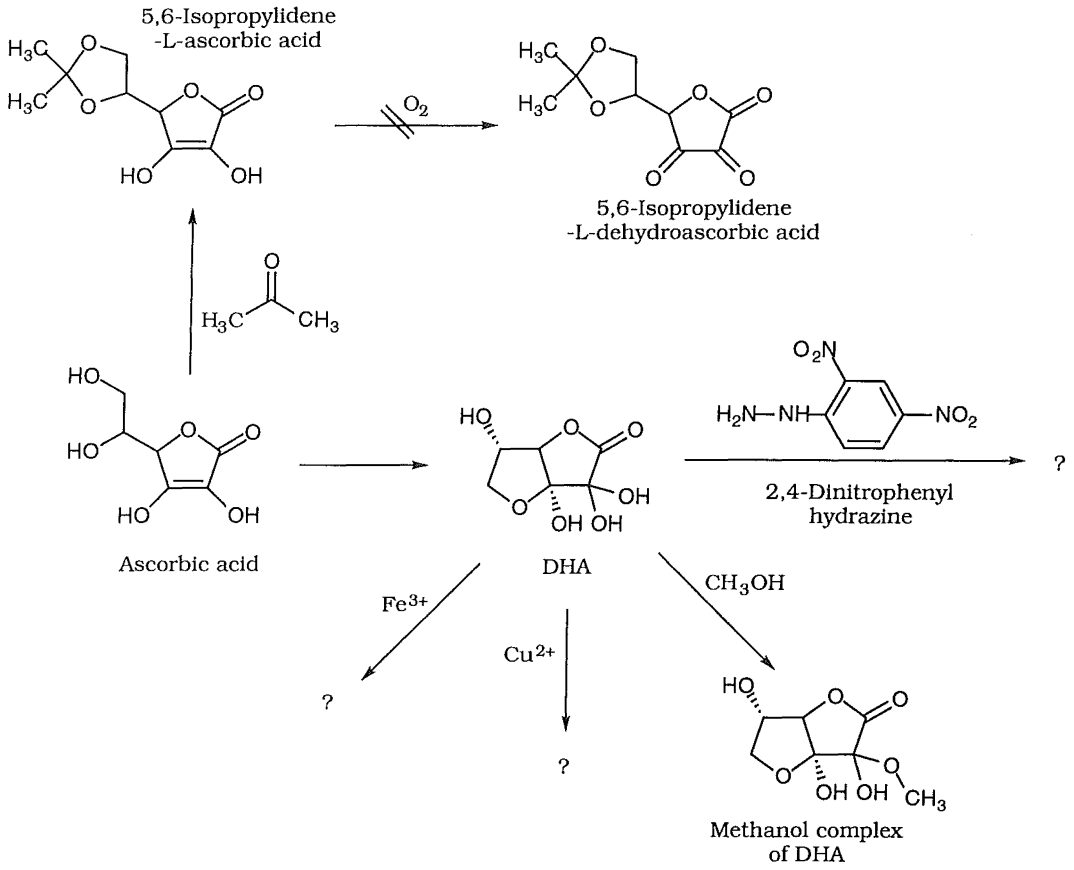


Figure 2.12: attempted preparation of DHA derivatives.

2.4.3 Stability and degradation products of dehydroascorbic acid

If stored below -10°C, the syrup form of DHA was found to be stable for over a year, as judged by ^{13}C and ^{1}H NMR. When dissolved in D_2O and kept at room temperature, however, it was found that degradation began after approximately twenty days. Complete loss of DHA occurred within thirty days. The main product shown in the ^{13}C NMR spectrum recorded at this time, was thought to be 2,3-diketogulonic acid (2,3-DKG), the hydrolysis product of DHA, as the ^{13}C NMR data matched that of a literature preparation.[38] Spectra

were not inconsistent with the minor presence of other compounds, such as threose.

A number of degradation products of DHA have been identified in the literature,[39-41] including 2,3-DKG,[42] threose,[43] oxalic acid,[44] and cyclotene,[41] as shown in *figure 2.13*. Others, such as glyoxal, can also be postulated as degradation products of DHA.[45] These products may also play a role in protein crosslinking. The relative importance of the breakdown products of ascorbic acid in the reaction with proteins, has not been established.

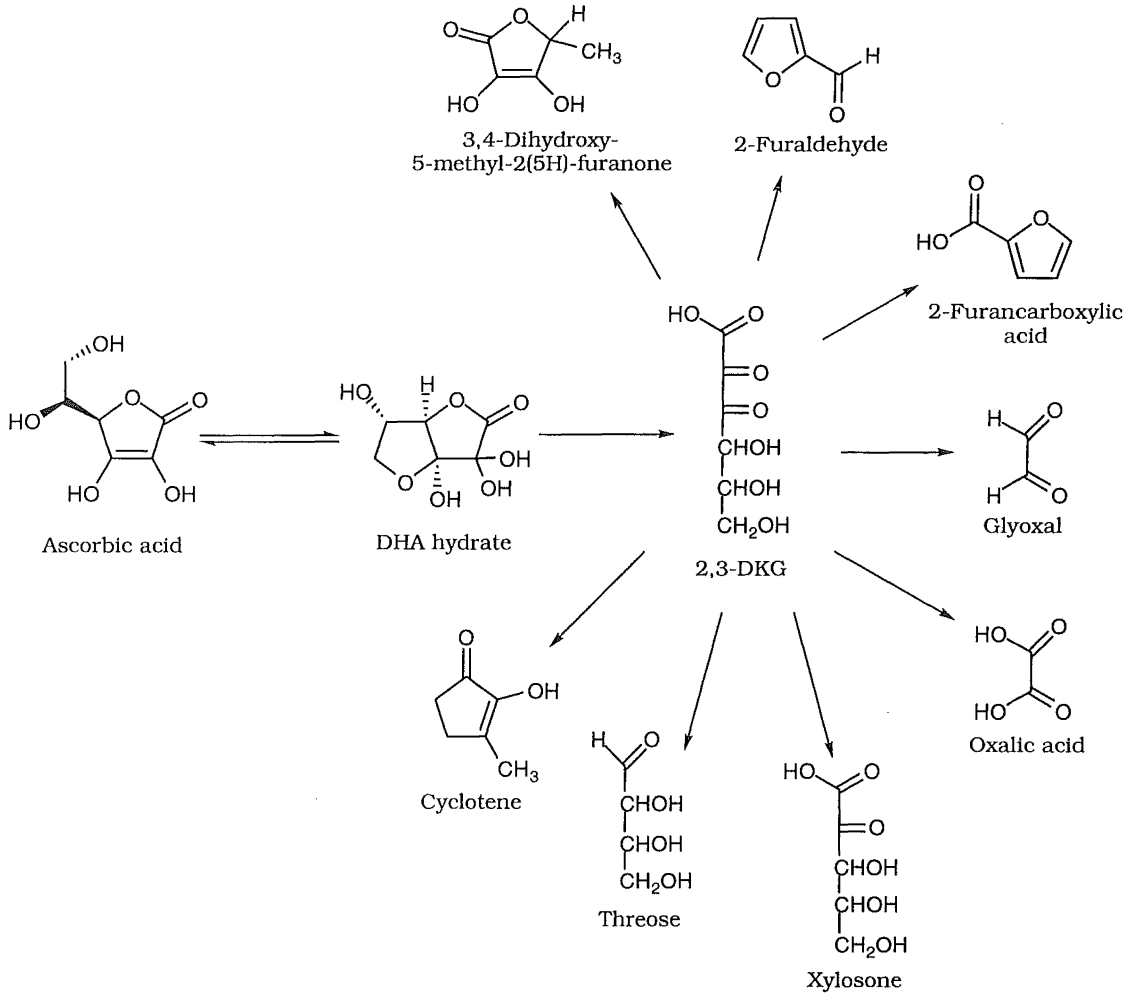


Figure 2.13: examples of degradation products of ascorbic acid.

Threose and 2,3-DKG have attracted some attention in this context.[42,44] Cyclotene has never been considered as a protein crosslinking agent; in fact, its general chemistry has been curiously ignored, despite its importance in foods and as a food additive.[46]

2.4.4 Characterisation of cyclotene

Cyclotene can, in principle, exist in many tautomeric forms, but is usually represented in either the dicarbonyl form or the enol form, shown in *figure 2.14*, since it displays reactions characteristic of both structures.

Figure 2.14: two tautomeric forms of cyclotene.

Due to the lack of information as to the structure of this compound, structural and spectroscopic studies were initiated in both solution and solid states.

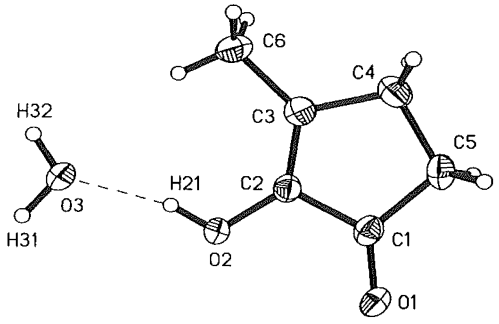


Figure 2.15: perspective view and atom labelling of cyclotene, 2-hydroxycyclopent-2-enone. Displacement ellipsoids are drawn at the 50% level and H atoms are drawn as small circles of arbitrary radius.

Cyclotene normally exists in a hydrated form, the nature of which had not been established. Therefore, solution NMR spectra were recorded (see *chapter eight*) and a single crystal X-ray structure determination was carried out (*figure 2.15*). This demonstrated for the first time that cyclotene, in fact, exists as the 2-hydroxycyclopent-2-enone tautomer (enol form of *figure 2.14*).[47] This structure determination proved useful for mechanistic studies presented in *chapter four.*

The cyclopentenone ring was found to be essentially planar and the compound's geometry similar to other crystallographically characterised cyclopentenones.[48]

The cyclotene hydroxyl group was shown to be hydrogen bonded to the oxygen atom of the adjacent water molecule. Each of the hydrogen atoms of the water molecule were found to be hydrogen bonded to the carbonyl oxygen atom of an adjacent cyclotene molecule, shown in *figure 2.16.* This network of hydrogen bonding has the effect of connecting the molecules in chains along the *b* axis.

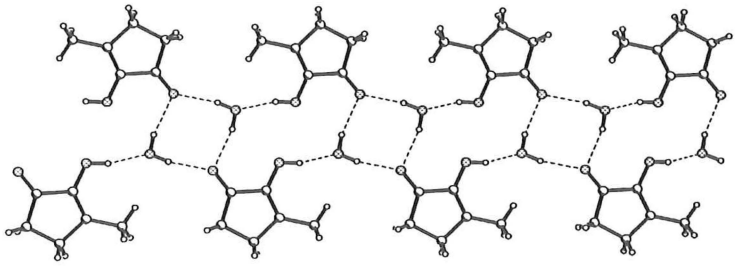


Figure 2.16: packing diagram viewed down the a axis showing the hydrogen bonding network.

2.5 Summary

As DHA was an ill-characterised compound, a method for the preparation of pure DHA was optimised and samples characterised by ^{13}C and ^{1}H NMR. Attempts to furnish crystalline monomeric DHA resulted in crystals that provided rotation photographs consistent

with crystalline material, but which were not of a sufficient quality to provide structure solution.

Various literature results were presented which support the supposition that initial nucleophilic attack, by an amino group, of the C2 carbonyl moiety of DHA, followed by attack at C3, may result in the formation of covalent crosslinks in a protein system. Further attempts to gain information about the structure and reactions of DHA by its derivatisation provided evidence of the complex nature of its reactions.

The stability of monomeric DHA was also examined. It was shown to be stable for at least a year when stored at -10°C, but degraded to compounds such as 2,3-DKG and threose, within a month, when dissolved in D_2O at room temperature. A degradation product, cyclotene, was investigated and its structure determined by X-ray crystallography. This work is in press.[47]

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THE REACTIONS OF DEHYDROASCORBIC ACID WITH AMINO ACIDS AND AMINO ACID DERIVATIVES

3.1 Background

The importance of protein crosslinking and how this may occur as a result of the Maillard reaction was discussed in *chapter one.* In *chapter two*, the chemistry of DHA was shown to be consistent with a molecule with high Maillard reactivity. This chapter will focus on the reactions of DHA with amino acids and simple derivatives.

Numerous studies have examined the Maillard reactions of sugars, such as xylose,[1] fructose,[2] galactose,[3] ribose[4] and glucose,[5] with amino acids. However, despite the importance of DHA as a breakdown product of ascorbic acid, a common food additive, and its importance in biological systems, very little is known about its reaction with amino acids and the structures of the resulting products.

At the outset of this work, a literature search revealed that few products of the reaction between DHA and amino acids had been identified. Those that had included a variety of coloured radical products, as well as low molecular weight compounds. The first part of this chapter reviews the reported chemistry of DHA with amino acids.

3.2 *Low molecular weight compounds formed by the reaction of dehydroascorbic acid with amino acids*

DHA is reported to react with amino acids to form a Schiff base which can then lead to the formation of low molecular weight compounds.[6] An example of a compound which has been identified from the reaction of DHA with amino acids is scorbamic acid (SCA).[7] It is believed to result from the Strecker degradation of amino acids, shown in *figure 3.1*.

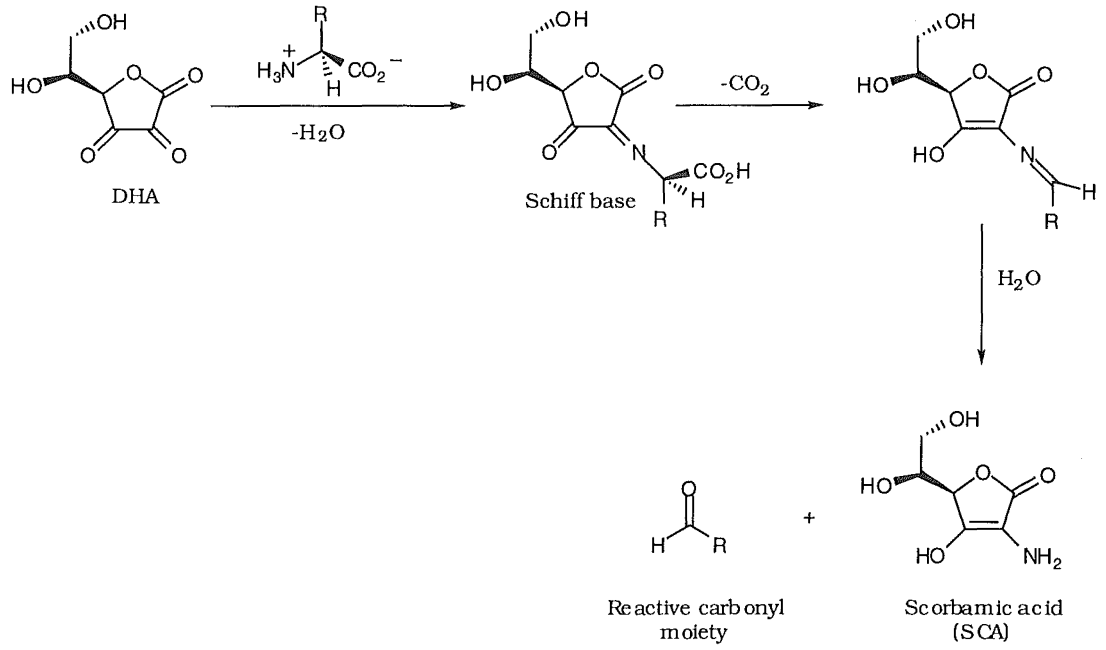


Figure 3.1: Strecker degradation of amino acids.[7]

SCA can condense with a second DHA molecule to give the red pigment 2,2'-nitrilodi-2(2')-deoxyascorbic acid. This pigment can be prepared by the reaction of DHA with any of a number of amino acids, since the amino acid R-group is eliminated during the reaction.[8]

The formation of this pigment, shown in *figure 3.2*, cannot lead to the production of covalent crosslinks directly. However, it provides evidence that DHA can readily form Schiff bases with amino acids. These Schiff bases have the potential to undergo alternate reactions, resulting in covalent crosslinks. The extent to

which this reaction occurs, and the nature of the other products of this reaction, have not been extensively studied.[6]

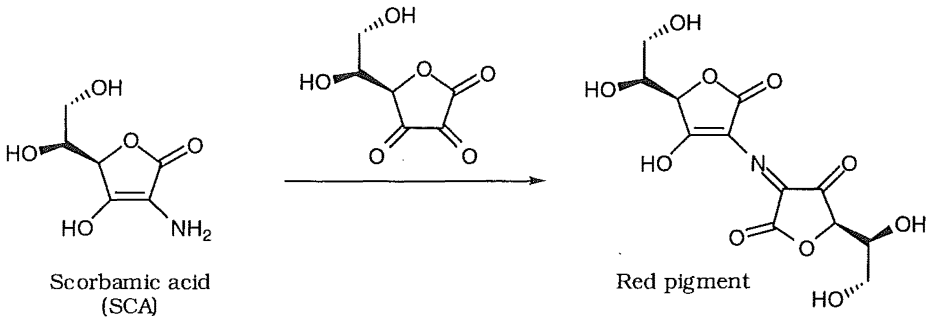


Figure 3.2: formation of a red pigment from scorbamic acid.[8]

3.2.1 *Reaction of dehydroascorbic acid with dipeptides and tripeptides*

Sakurai and co-workers have investigated the reactions of DHA with various glycyl dipeptides and tripeptides.[9-12] Saturated, methanolic solutions of the peptides were refluxed with DHA for thirty minutes. The solutions were then concentrated and a series of ninhydrin positive substances, separated by thin layer chromatography (TLC), were isolated as disodium salts, from non-aqueous solvents, for each reaction. Although the products of these reactions do not contain free amino groups, subsequent imine hydrolysis gives the compound SCA, which does contain a free amino group and, therefore, is ninhydrin-positive.

The structures of the isolated disodium salts were then determined using mass spectrometry (MS), ultraviolet (UV), infrared (IR) and NMR spectroscopy. The products of the reaction of DHA with glycyl dipeptides were shown to be the glycyl-DL-leucine disodium salt derivative, and the glycyl-DL-alanine disodium salt derivative. The structures of a number of other compounds detected by thin layer chromatography (TLC) were also suggested to be glycyl dipeptide derivatives. A possible pathway for the formation of these ninhydrin-positive substances is shown in *figure 3.3.*[9-12] Neither further reaction products nor other reaction conditions were studied.

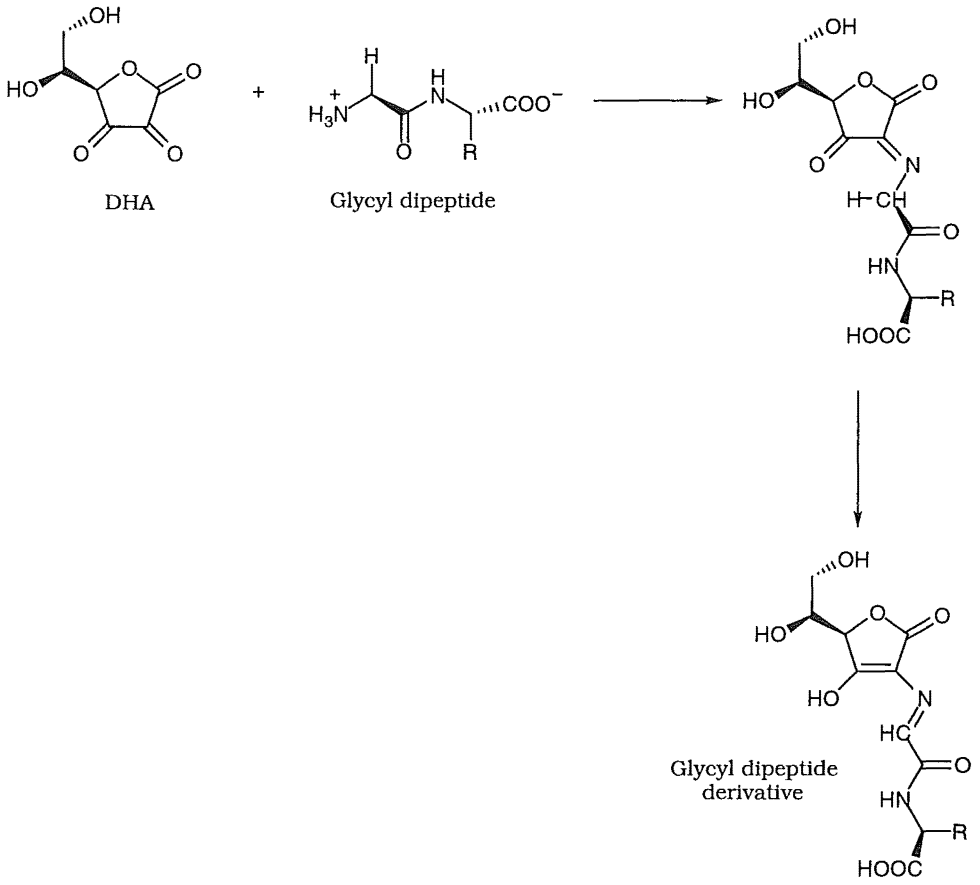


Figure 3.3: possible reaction pathway for the formation of ninhydrin positive substances from the reaction of DHA with glycyl dipeptides.[9-11]

3.2.2 *Radical products formed by the reaction of dehydroascorbic acid with amino acids*

The reaction of DHA with an amino acid has been found to generate several coloured free radical products.[13] The products were prepared by refluxing DHA with an amino acid in 95% ethanol and the stable products were then isolated by preparative TLC. Their identification was based mainly on the hyperfine structures of their electron spin resonance (ESR) signals and from their UV, IR and NMR spectra.

Examples of the products isolated include a variety of radical species which are believed to be related to the red pigment mentioned above.[14] Regardless of which amino acid is used to prepare the red species, no change in their ESR signal has been

observed. This observation provides further evidence that the R-group of the amino acid has been eliminated, and that the formation of the pigment, and its associated radicals, is likely to be *via* the Strecker degradation.[15]

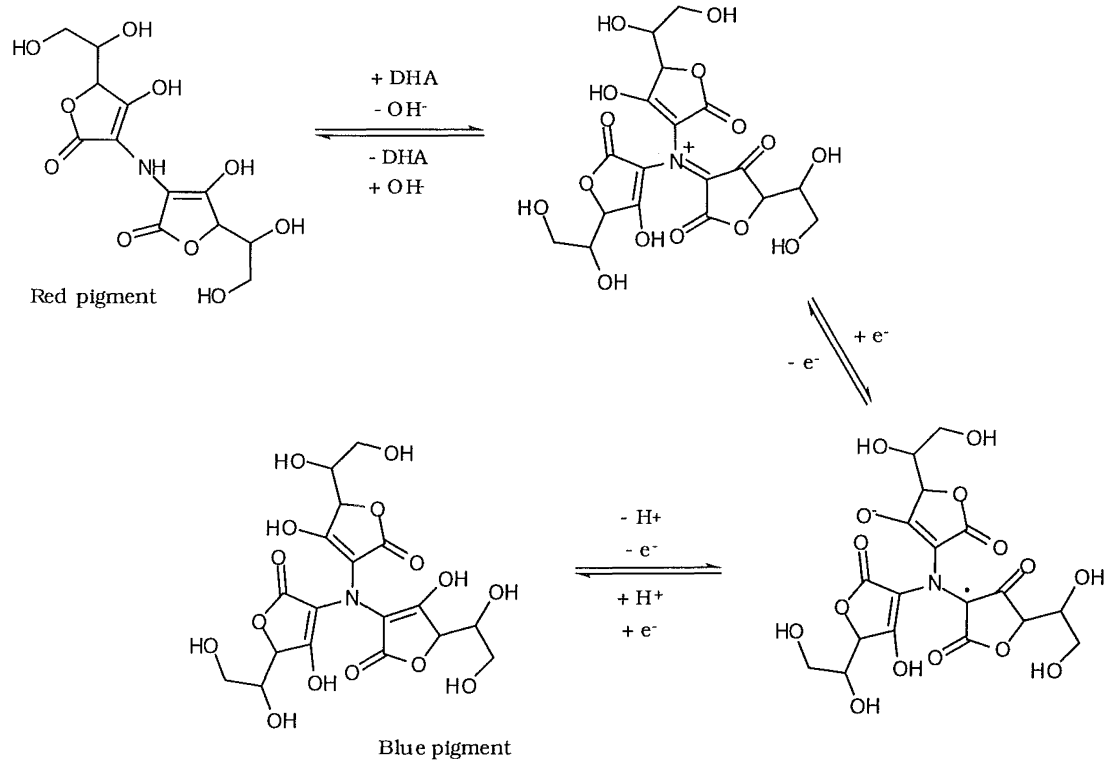


Figure 3.4: proposed structures of the blue pigment, its associated radical products and their relationship with the red pigment.[13]

A number of other radical species are also believed to be related to the red pigment. These include yellow[16] and blue pigments. The blue pigment has been identified as tris-(2-deoxy-2-L-ascorbyl)amine, as shown in *figure 3.4.*[13] A purple pigment has also been detected in the reaction of DHA with phenylalanine. This pigment is believed to have retained the phenylalanine R-group and, therefore, is not formed *via* the Strecker degradation.[17]

3.2.3 *Reaction of the degradation products of dehydroascorbic acid with amino acids.*

In addition to the direct reaction of DHA with amino acids, a number of compounds have been isolated from the reaction of amino acids with various breakdown products of DHA. Examples of these include N-ε-(carboxymethyl)lysine (CML) and 3-(N-ε-lysino)-lactic acid (LL). It has been proposed that these compounds are formed by the reaction of threose, a degradation product of DHA, with a lysine residue. The resulting Schiff base then rearranges to give the Amadori product, an aminoketose, followed by oxidative cleavage to give CML and LL as shown in *figure 3.5.*[18]

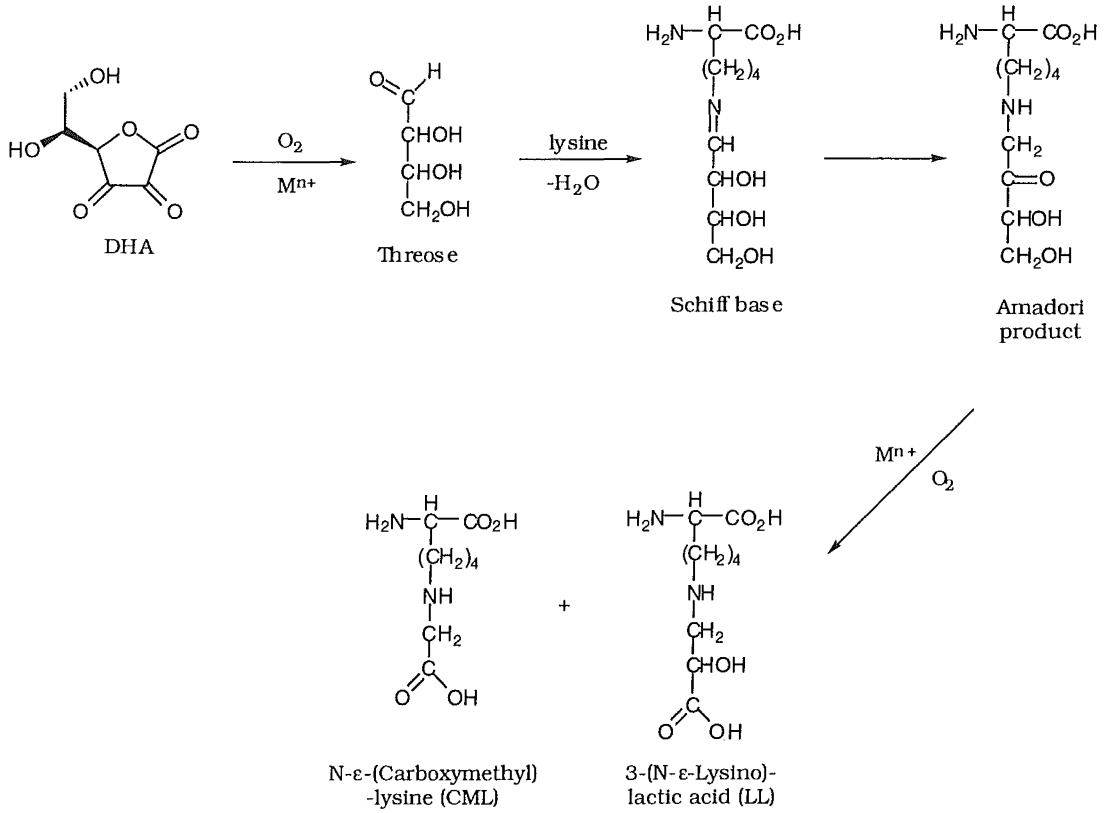


Figure 3.5: possible mechanism for the formation of two low molecular weight products from the reaction of DHA with lysine.[18]

Further investigations into the formation mechanism of CML suggest that it could also be formed by the reaction of lysine with DHA itself. The mechanism proposed in *figure 3.6* may account for the fact that CML is obtained in a much greater yield when DHA is

reacted with lysine compared with that from the reaction of threose with lysine.[19] There is some debate as to which reaction mechanism is favoured and under what conditions. It is, therefore, important to study the reactions of breakdown products of DHA with proteins, as well as those of DHA itself, in order to elucidate the various reaction mechanisms.

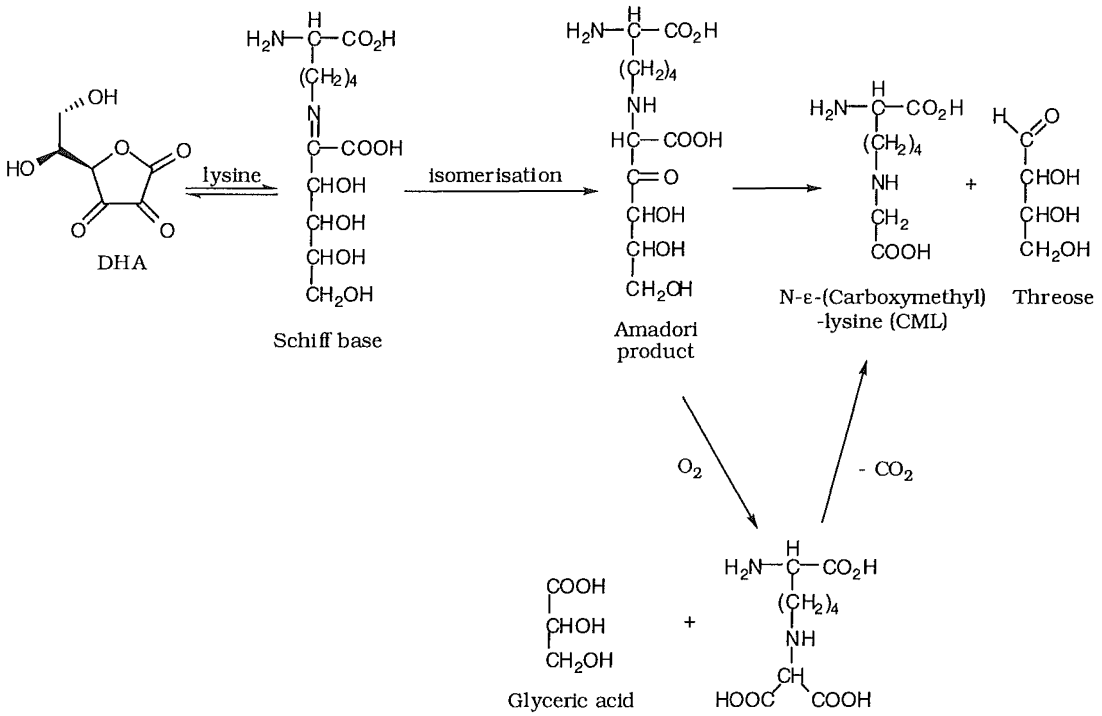


Figure 3.6: suggested alternative reaction pathway for the formation of CML.[19]

Further examples of products isolated from the reaction of degradation products of DHA with amino acids include 3-deoxythreosone (3-DT) and formyl threosyl pyrrole (FTP). Both compounds have been isolated from the reaction of threose with lysine.[20,21]

A suggested mechanism for the formation of FTP is shown in *figure 3.7.* The synthesis is believed to occur by the reaction of the Amadori product of threose-lysine with a second product from the reaction of threose with N-α-acetyllysine, 3-DT.

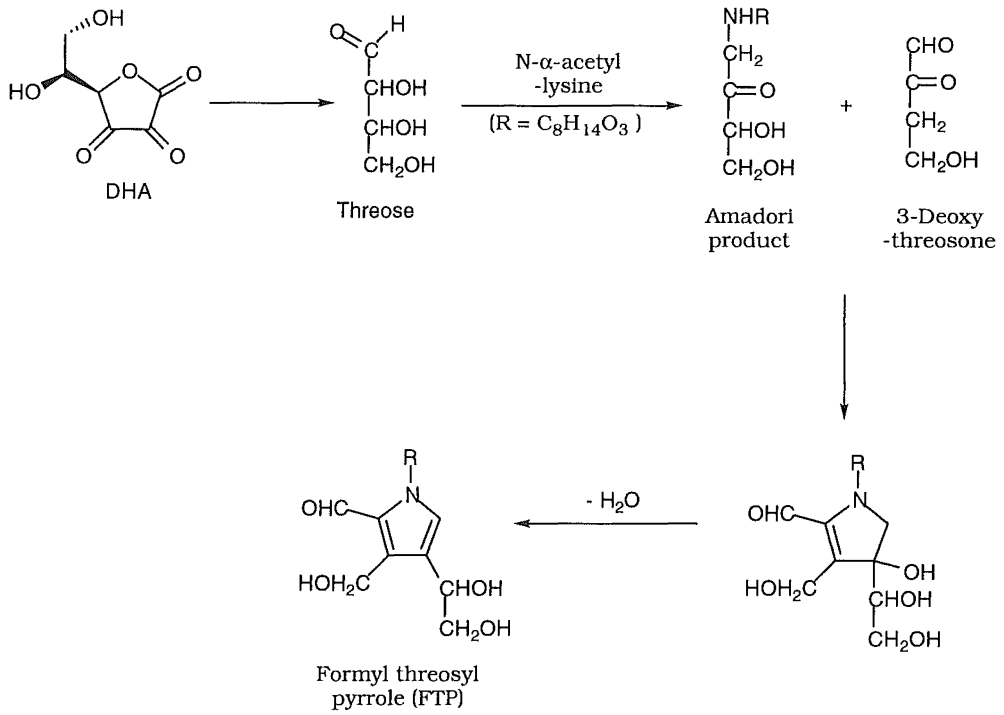


Figure 3.7: proposed mechanism of formation of FTP.[21]

As can be intimated from the above literature survey, a large number of products from the reaction of DHA with amino acids remain uncharacterised. Therefore, by exploring the reactions of DHA with individual amino acids, we hoped to gain some insight into the nature of the interaction of DHA with proteins, which are much more complicated systems.

3.3 *Comparison of the Maillard reactivity of dehydroascorbic acid with that of xylose*

The Maillard reaction is also commonly referred to as the browning reaction since the development of colour is one of its most obvious effects. The rate of the Maillard reaction is often monitored by recording the absorbance of the reaction mixture at 460 nm.[22] Although this rate measurement only provides an approximation of the relative rates of reaction, since it is not measuring the development of single (or common) chromophores,[22] it is commonly used to compare the rates of different amino acid or sugar systems.[23]

As not much was known about the Maillard reactivity of DHA with amino acids, we began by adopting methodology from the better characterised amino acid-sugar systems,[23] and comparing the reactivity of DHA and lysine and ascorbic acid and lysine, with that of xylose and lysine, which is better characterised.[24] Ascorbic acid was used as the control in this series of reactions, to confirm that the browning reaction was due to DHA, and not an equilibrating mixture of DHA and ascorbic acid.

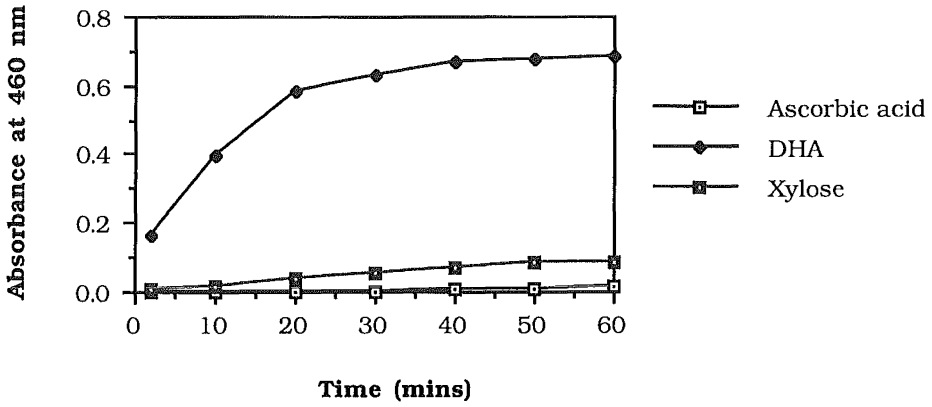


Figure 3.8: Comparison of the reaction between DHA and lysine, xylose and lysine and ascorbic acid and lysine, as judged by absorbance at 460 nm.

In *figure 3.8*, DHA can be seen to be a much more reactive Maillard reagent than xylose under these conditions, and very much more reactive than ascorbic acid. It is proposed that ascorbic acid can only take part in Maillard reactions after its oxidation to DHA and that the slow rate of browning reflects the requirement for this oxidation.

This investigation was extended to include the amino acids glycine and N-α-*t*BOC-lysine, which provide a better model for an actual protein system, where the α-amino group is involved in the peptide bond.

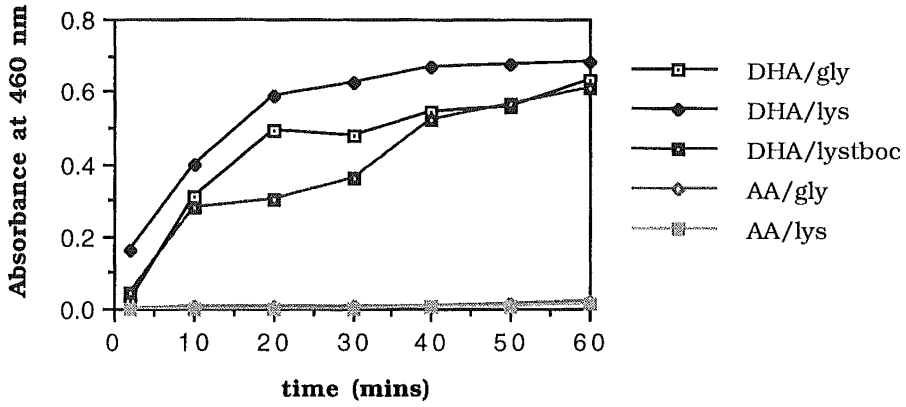


Figure 3.9: comparison of the reactions of DHA and ascorbic acid with amino acids by monitoring at 460 nm.

As can be seen in *figure 3.9*, DHA was the much more reactive Maillard reagent under these conditions. Its reaction with glycine and lysine produced a rapid increase in colour compared with the reactions of ascorbic acid, which produced no visible colour change.

The reaction of ascorbic acid with N-α-*t*BOC-lysine resulted in the formation of a dark brown oil within minutes of reaction. The reason for this is not clear.

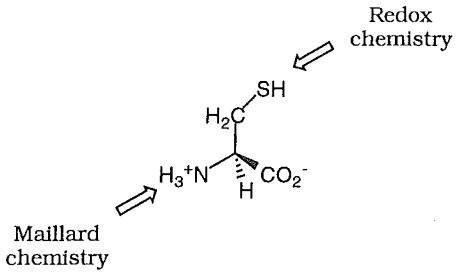


Figure 3.10: competition between redox chemistry and Maillard chemistry for the amino acid cysteine.

The reaction of DHA with the thiol-containing amino acid cysteine was also investigated as, in proteins, the cysteine residue is believed to be oxidised by DHA, forming a disulfide crosslink.[25] Therefore, redox chemistry at the thiol group competes with

Maillard chemistry at the α-amino group of the amino acid cysteine, as shown in *figure 3.10.*

The reaction of DHA with cysteine resulted in the oxidation of cysteine to the disulfide cystine, which immediately precipitated. This was confirmed by NMR, which showed the presence of both cystine and ascorbic acid, the reduction product of DHA. This observation demonstrates that in the case of free amino acids, under these conditions, disulfide bonding is the predominant chemistry. The formation of the disulfide bonded cystine, results in the precipitation of the amino acid. The amino groups of cystine are, therefore, no longer in solution and available for Maillard chemistry. Because of this, the relative rates of the redox chemistry versus Maillard chemistry cannot be compared, although, clearly, redox chemistry predominates.

In a protein system, however, the hydrophobic cysteine residues will reside in the centre of the protein molecule making them less available for reaction.[26] The lysine residues, on the other hand, are hydrophilic and, hence, will reside on the outside of the folded protein molecule and be readily available for reaction. Also, unlike cystine, proteins containing disulfide bonding are able to remain in solution, so are vulnerable to both Maillard and redox chemistry. Hence, it was expected that the comparative rates of reaction between disulfide chemistry and Maillard chemistry, involving the lysine residue, would be quite different in a protein system and required investigation.

3.4 Attempted characterisation of the dehydroascorbic acid-amino acid reaction products

The above results demonstrate that DHA, or its breakdown products, are likely to play a major role in the degradation of the amino acid lysine. We believe this to occur through the formation of Schiff bases, which, in turn, may lead to the formation of covalent crosslinks. To investigate this further, DHA was reacted with various amino acids with a view to separating and characterising the products.

3.4.1 Preparation of dehydroascorbic acid-amino acid systems

Methodology developed by Sakurai *et al.*, was adopted for the preparation of the DHA-amino acid reaction products.[9-12] This involved the reflux of a methanolic solution of DHA with an equimolar amount of a specific amino acid for forty-five minutes. To compare the reaction of Sakurai *et al.* with conditions closer to those of food processing and physiological conditions, the experiments were also carried out in aqueous solution. A comparison of the two reaction methods, for each amino acid, was achieved with the use of TLC. Minor differences in the observed product ratios, with the change in solvent, were found, but no differences in the products themselves.

The reaction mixtures were run on three separate plates, in parallel, using an *n*-butanol-ethanol-water solvent system. The first of the three plates was used to investigate both UV and visible products. The second plate was sprayed with ninhydrin reagent in order to visualise compounds containing free amino groups. The reaction of these groups with the ninhydrin appeared as a purple spot after spraying. The last of the plates was used to visualise compounds containing carbonyl groups, such as DHA, by spraying with 2,4-dinitrophenylhydrazine. The hydrazine reacts with any aldehyde or ketone groups present producing a red-orange spot.

As can be seen in *table 3.1*, both unreacted amino acid and either DHA or ascorbic acid are present in each of the reaction mixtures. Unfortunately, as pure solutions of DHA or ascorbic acid quickly re-equilibrate, they had the same Rf value and were, therefore, indistinguishable. The formation of a red coloured product was also observed in each of the reaction mixtures. No other separated products were visualised. Extensive attempts to improve the separation of the products by varying the ratios of the components of the solvent system did not result in greater resolution.

Rf Values	0.06	0.14	0.34	0.36	0.45	0.55	0.65	0.77
DHA/AA							DNP	
glycine			NPS					
DHA-glycine			NPS			Vis.	DNP	
DHA/AA							DNP	
cysteine		NPS			NPS			
cystine		NPS			NPS			
DHA-cysteine		NPS			NPS	Vis.	DNP	
DHA/AA							DNP	
lysine	NPS			NPS				
DHA-lysine	NPS			NPS		Vis.		
lysine-α-CBZ								NPS
DHA-α-lysine-CBZ						Vis.	DNP	NPS
lysine-α-tBOC								NPS
DHA-lysine-α-tBOC						Vis.	DNP	NPS

Table 3.1: TLC results of DHA-amino acid systems in either water or methanol. Spots are designated NPS (ninhydrin positive spot), DNP (2,4-dinitrophenylhydrazine positive spot) and Vis. (visible spot - red).

As many more products had been expected, reversed-phase high performance liquid chromatography (RP-HPLC) was employed to gain a clearer picture of the components of the reaction mixture. We hoped to separate and collect the components of each DHA-amino acid reaction mixture which would, ultimately, lead to their characterisation.

3.4.2 *Separation of the dehydroascorbic acid-amino acid reaction products using reversed-phase high performance liquid chromatography*

As the products of the reactions of xylose and lysine have been separated by RP-HPLC and are relatively well-characterised,[23] this system was compared to the reaction of DHA with lysine.

In each case, the starting materials were dissolved in methanol and the stirred solutions were refluxed for forty-five minutes. The solvent was then removed and a known quantity of the dried products was redissolved for analysis using the RP-HPLC method developed by Bailey *et al.* for the separation of xylose-amino acid reaction mixtures.[23]

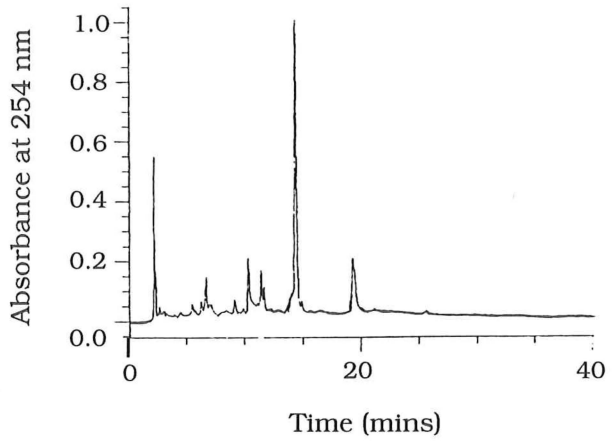
When the xylose-lysine reaction mixture was separated using this procedure, it contained unretained peaks consisting of material excluded from the column, either on the basis of molecular size or polarity, unresolved material running as a convex broad band and unresolved material running as a tailing broad band. It has been suggested that these products may be polymer molecules which are unable to equilibrate with the stationary phase of the column.[23]

Resolved peaks were also obtained, although the areas of these peaks were small relative to the areas of the broad bands and unretained peaks. These results are consistent with those obtained by Bailey *et al.*[21]

In contrast, the DHA-lysine system had shown much greater reactivity, in initial studies. Its HPLC chromatogram contained mainly unresolved peaks, at retention times only slightly greater than that of the exclusion volume, and a tailing broad band, suggesting that a different separation system was required (see *figure 3.11*).

A new method was developed to try and effect better separation of the DHA-lysine model system. This involved the use of either a C_8 or a C_{18} reversed-phase analytical column with 25% methanol as the eluting solvent.

a) RP-HPLC separation of the products of the xylose-lysine reaction system.



b) RP-HPLC separation of the products of the DHA-lysine reaction system.

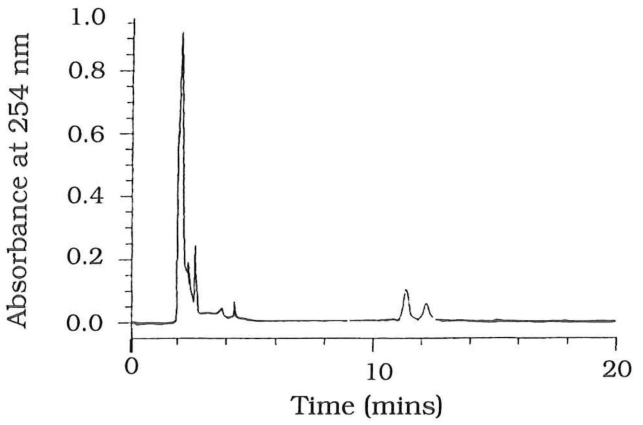


Figure 3.11: typical comparison of the reaction products from the DHA-lysine reaction system and the xylose-lysine reaction system as judged by HPLC analysis. (Note expanded scale in 3.11b).

Improved resolution was obtained by this method (*figure 3.12*) and was sufficient to allow identification of two products, ascorbic acid, which was identified by comparison of its retention time with that of an authentic sample, and the red pigment, identified by comparison of its UV trace to that reported in the literature for this compound.[27] This resolution, however, was difficult to reproduce. Best results were obtained with the use of an older

HPLC column, possibly due to an increase in the polarity of the column.[28]

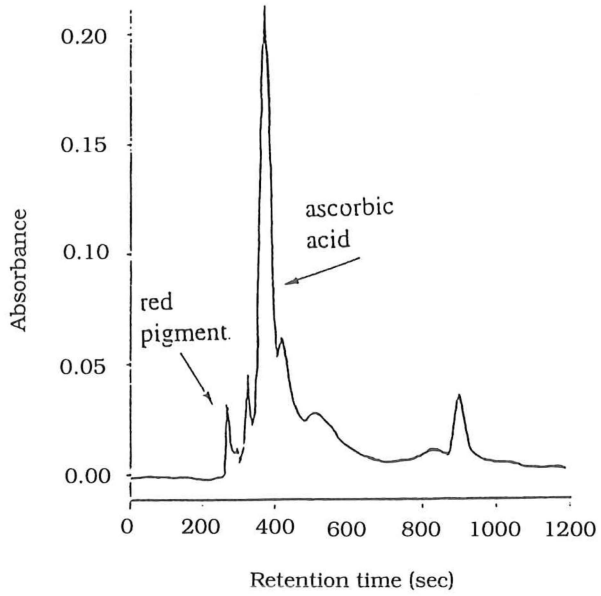


Figure 3.12: improved resolution in the separation of the products from the DHA-lysine reaction model system. Absorbance was monitored at 203 nm.

3.5 *Compounds recently isolated from the reaction of dehydroascorbic acid with amino acids*

Research by Pischetsreider *et al.*, which has occurred contemporaneously to that described in this thesis, has resulted in the identification of a number of other products from the reaction of DHA with amino acids in a pH-controlled system.[29-33]

Commercial DHA was used for this series of reactions and was purchased from Sigma-Aldrich. This product contains large quantities of decomposition products, as shown in *chapter one.* Thus, some of the reported reaction products may result from impurities in the starting material.

The products of the reactions were separated and collected using HPLC with excess potassium dihydrogen phosphate, which had been acidified to pH 3 with phosphoric acid, and methanol as the solvent system. Through the use of this acidic solvent, the HPLC

trace can be simplified considerably since a number of the reaction products are known to be acid-labile.[34] This simplification leads to improved resolution of the remaining products, and increases the ease with which they can be collected.

In contrast, freshly prepared, monomeric DHA was used for all of the amino acid reaction systems described above, which were carried out without pH control. By examining the more complex reaction systems, we hoped to gain as much insight as possible into the reaction of DHA with amino acids. The resulting product mixture, however, proved to be too complex to separate by standard techniques.

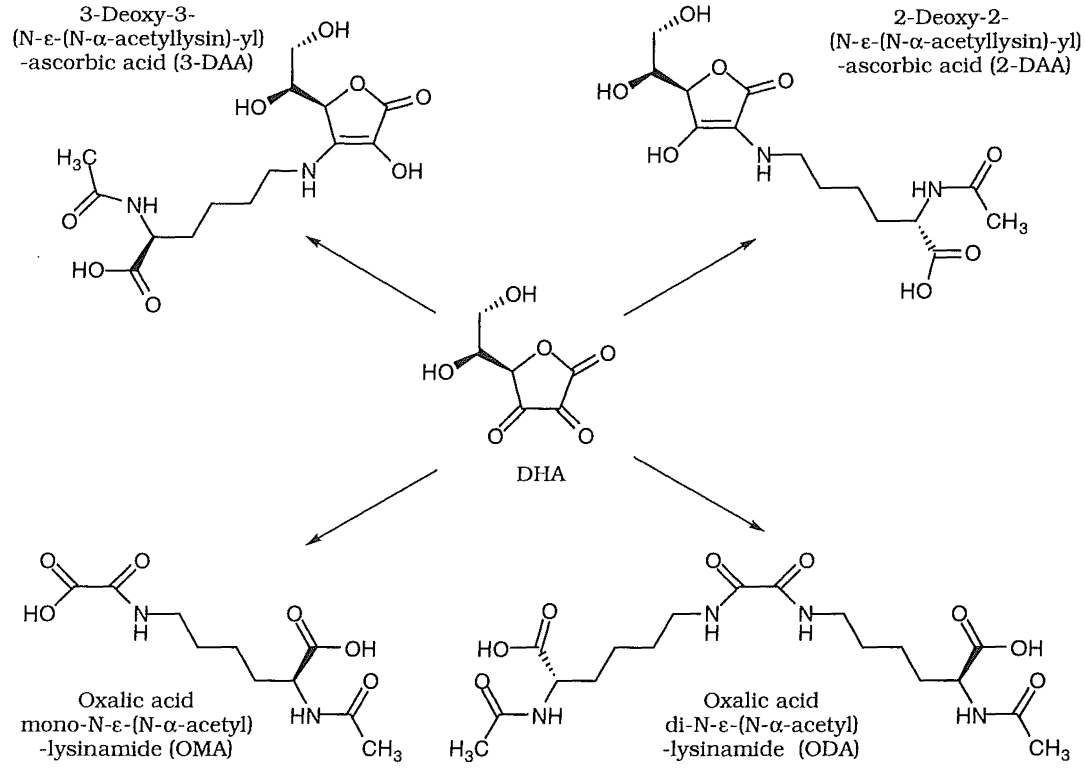


Figure 3.13: four products identified from the reaction of DHA with N-α-acetyllysine.[30]

Pischetsreider and co-workers investigated the reactions of DHA with the amino acids N-α-acetyllysine and N-α-acetylarginine. The major products isolated from the former reaction were found to be 2-deoxy-2-(N-ε-(N-α-acetyllysin)-yl)-ascorbic acid (2-DAA), oxalic acid mono-N-ε-(N-α-acetyl)lysinamide (OMA) and oxalic acid di-N-

ε-(N-α-acetyl)lysinamide (ODA), shown in *figure 3.13.* Further minor products were identified as 3-deoxy-3-(N-ε-(N-α-acetyllysin)-yl)-ascorbic acid (3-DAA) as well as regenerated ascorbic acid.

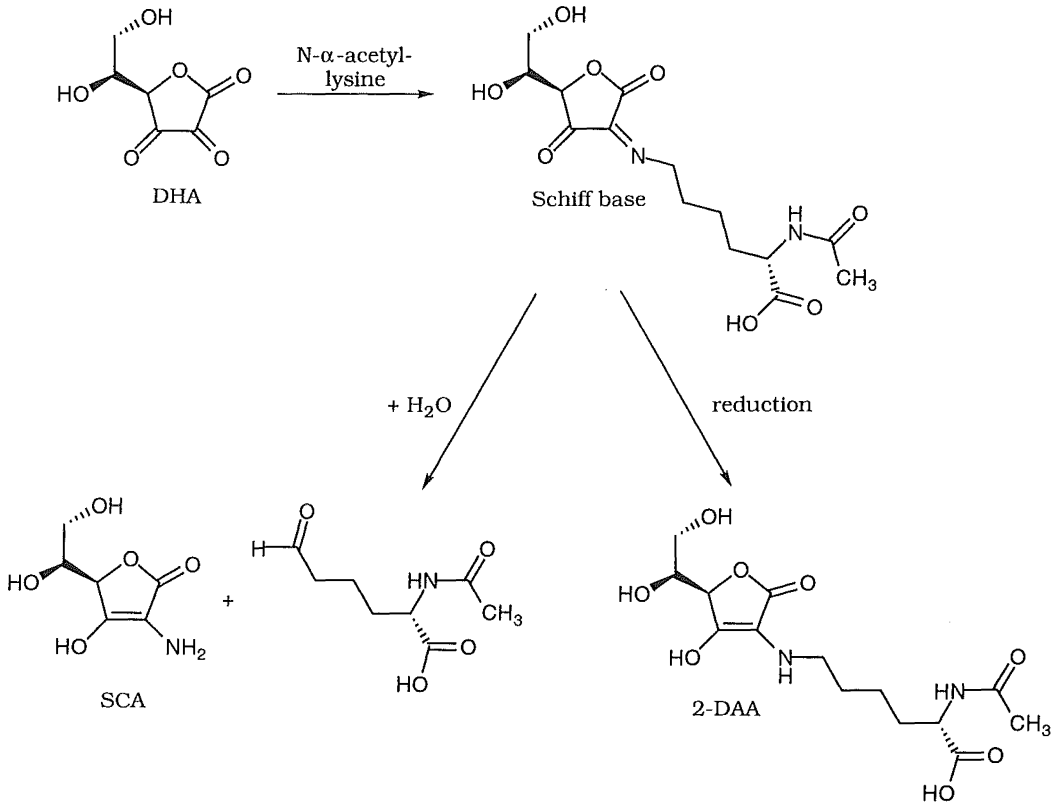


Figure 3.14: reaction of DHA with N-α-acetyllysine to give either 2-DAA or SCA.[30]

The formation of the product 2-DAA has been assumed to occur *via* the formation of a Schiff base at the C2 carbonyl group of DHA, which is then reduced to give 2-DAA. Alternatively, the Schiff base intermediate can undergo deprotonation to give SCA which has been discussed previously (*figure 3.14*). In the conditions studied by Pischetsreider and co-workers, however, SCA formation was not favoured and, therefore, was not detected in the reaction mixture.[30]

The compound 3-DAA is also believed to form *via* Schiff base formation, this time at the C3 carbonyl group, which undergoes reduction, giving 3-DAA. An alternative mechanism proposed,

shown in *figure 3.15*, involves the reaction of N-α-acetyllysine with regenerated ascorbic acid.

The presence of compounds such as OMA and ODA in the reaction mixture may represent a mechanism by which two protein molecules can be crosslinked. To investigate whether this form of product occurs in a protein system, Pischetsreider *et al.* developed an immunochemical method for the detection of OMA as an 'ascorbylation' product of proteins.[30] Polyclonal antibodies were produced against an authentic sample of OMA protein. These were then used to demonstrate the presence of OMA modified protein in ascorbic acid-protein systems which had been incubated for two weeks. As OMA was only formed under aerobic conditions, it was assumed that the ascorbic acid must be oxidised to DHA prior to the formation of OMA modified proteins. The mechanistic details of this process have yet to be elucidated.

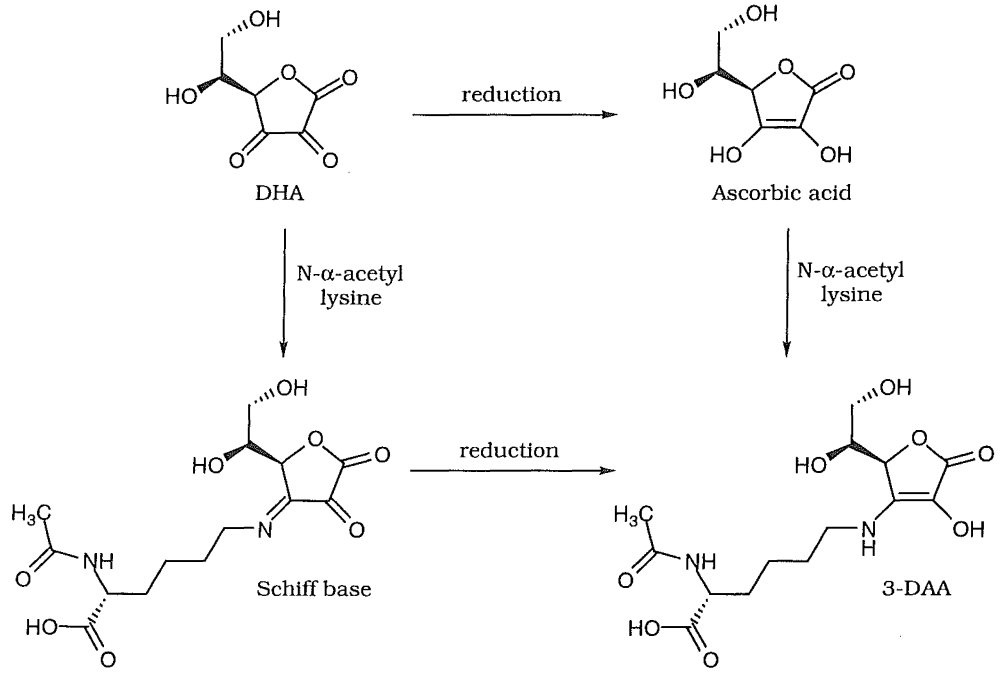


Figure 3.15: alternative reaction mechanisms for the formation of 3-DAA.[30]

When N-α-acetyllysine is replaced by the amino acid N-α-acetylarginine, a further reaction product, shown in *figure 3.16*, N-α-acetyl-δ-(4-(1,2-dihydroxy-3-propyliden)-3-imidazolin-5-on-2-

yl)-L-ornithine (DPI), is detected.[33] To form DPI, ascorbic acid is thought to be oxidised to DHA and then decarboxylated to give xylosone. The α-dicarbonyl group of xylosone can then react with the guanidine residue of N-α-acetylarginine forming an imidazoline derivative which can dehydrate to give DPI. This pathway has been assumed, since similar reactions are known to occur with a number of dicarbonyl compounds.[35,36]

This product has been assumed to be responsible for the loss of arginine during the incubation of proteins with either ascorbic acid or DHA.

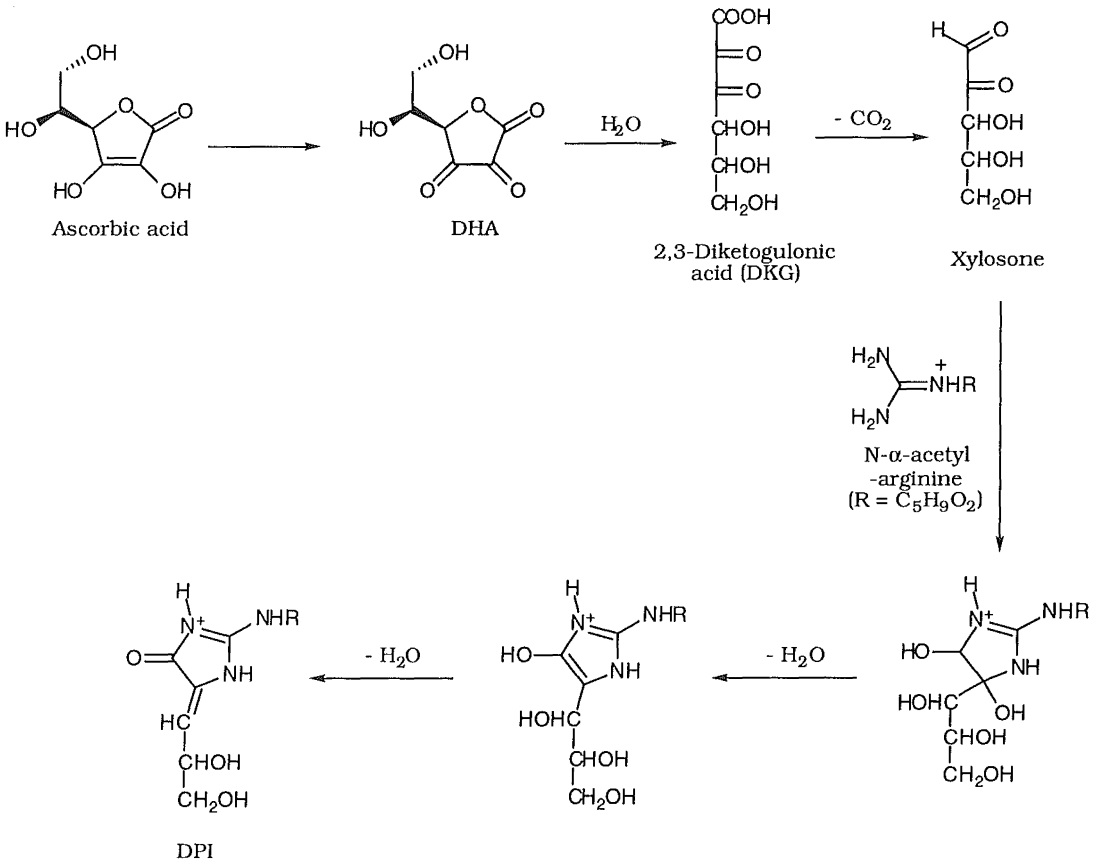


Figure 3.16: mechanism of formation of N-α-acetyl-δ-(4-(1,2-dihydroxy-3-propyliden)-3-imidazolin-5-on-2-yl)-L-ornithine (DPI).[33]

3.6 Summary

A review of the literature demonstrated that the free amino groups of α-amino acids are able to react at both the C2 and C3 carbonyl groups of DHA, producing a variety of products, as summarised in *figure 3.17.* These include compounds such as CML and FTP, as well as a range of coloured radical species.

We have shown that the Maillard reactivity of DHA is very much greater than that of both ascorbic acid and xylose, when reacted with a variety of amino acids. An HPLC method was then developed for the separation of the products of the reaction of DHA with amino acids. The improved resolution of this method allowed the identification of two products, ascorbic acid and 2,2'-nitrilodi-2(2')-deoxyascorbic acid.

A number of products, from the reaction of DHA with N-α-acetyllysine, were concurrently separated and identified by Pischetschreider *et al.* In this case, separation was achieved by an HPLC method using an acidic solvent system, thus greatly simplifying the chromatogram through the loss of acid-labile products.

These results support our hypothesis that free amino groups, such as that of the amino acid lysine, are capable of reacting at both the C2 and C3 carbonyl moieties, which may lead to protein crosslinking.

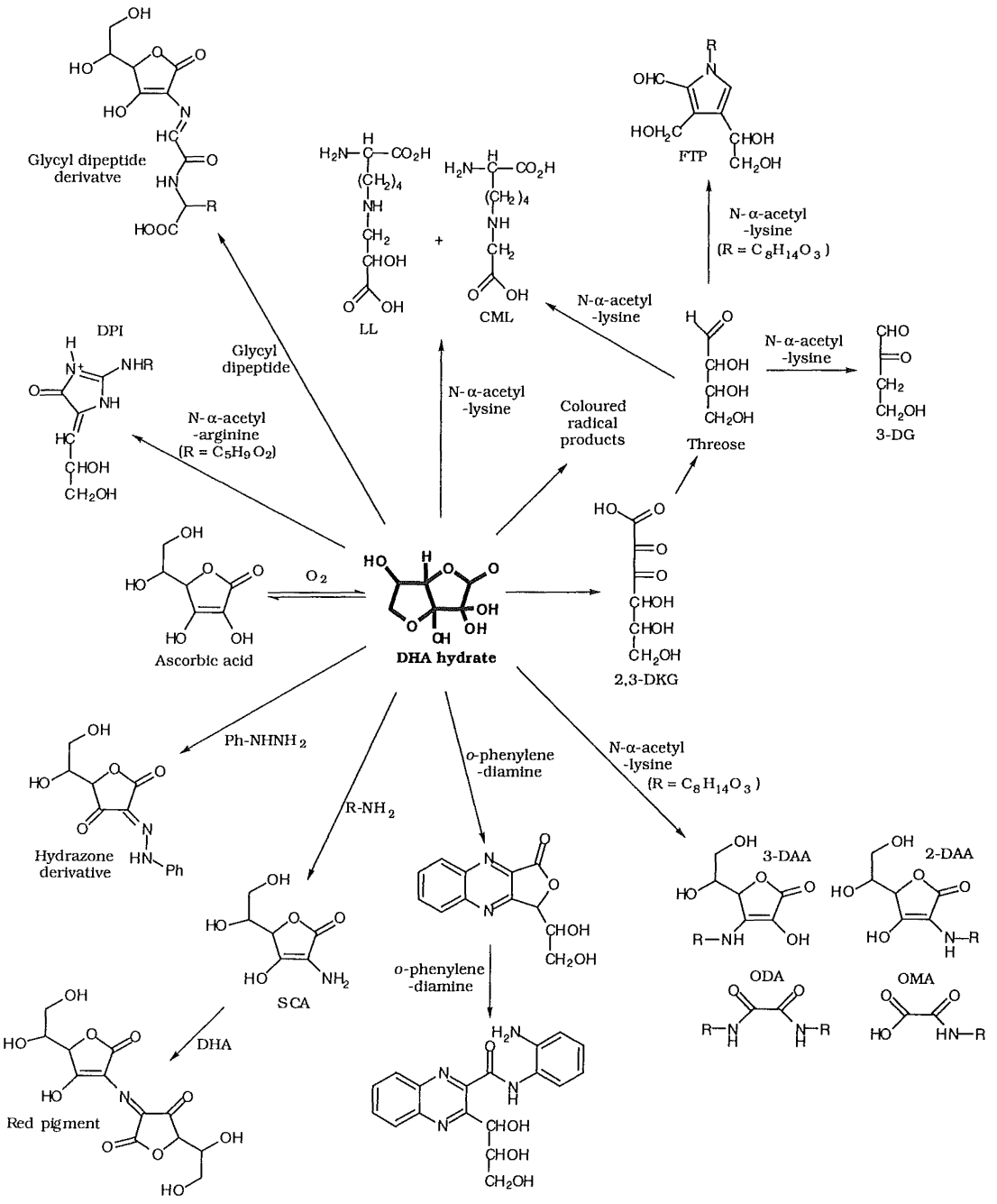


Figure 3.17: examples of the variety of products formed when DHA reacts with free amino groups.

3.7 References

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PROTEIN CROSSLINKING MEDIATED BY DEHYDROASCORBIC ACID

4.1 Background

Having discussed the importance of protein crosslinking resulting from the Maillard reaction, and examined the chemistry of DHA and its reactions with amino acids, the next stage of this research was to investigate the reactions of DHA in protein systems.

It has been assumed[1] that DHA forms disulfide crosslinks in biological systems, oxidising cysteine residues and being converted to ascorbic acid. However, we propose that DHA may also mediate the formation of non-disulfide crosslinks.

The formation of protein crosslinks, *via* the Maillard reaction of sugars, is important in food systems, where the crosslinking of proteins can affect the structure and properties of the protein.[2] Crosslinking can also result in the decreased nutritional quality of food products, through the destruction of essential amino acids and a decrease in digestibility.[3]

Although ascorbic acid, and its oxidation product DHA, are commonly found in food products, little is known about the Maillard reactions of these compounds in foods, how they may affect functional properties of proteins and whether this may lead to non-disulfide crosslinking. The relative importance of these two possible mechanisms for protein crosslinking has not been established. In fact, the possibility of DHA reacting with proteins *via* the Maillard reaction has been barely studied in food systems.[4]

The reaction of ascorbic acid derivatives with proteins has been recognised in the medical area. Even here, the only well-studied Maillard protein crosslinking system is that leading to the formation of age-onset, or diabetic cataract, during which the proteins of the eye, which are particularly long-lived, have been shown to undergo non-disulfide crosslinking.[5] Research in this area was initially based on the reactions of glucose with the crystallin proteins, the structural proteins of the lens, as significantly elevated levels of glucose can be present in the diabetic lens.[6] Ascorbic acid is also present, in both diabetic and normal lenses, in unusually high concentrations, where it is thought to protect against oxidative damage.[5] Therefore, ascorbic acid and its derivatives are also available to participate in Maillard type chemistry within the lens. Prabhakaram *et al.*[7] have shown that ascorbic acid can cause extensive crosslinking of the lens proteins, under aerobic conditions. The oxygen requirement of this reaction is believed to reflect the need for the initial oxidation of ascorbic acid, to products such as DHA, prior to the formation of protein crosslinks.

Several studies have since demonstrated that DHA and its breakdown products, DKG and threose, are also capable of crosslinking lens proteins when incubated at 37°C in aqueous solution.[8-10] It is not clear which of these compounds is responsible for the formation of crosslinks, or by what mechanism this occurs.

Each of these studies investigated the crosslinking reactions, of the protein crystallin, under physiological conditions. Protein crosslinking may also occur with other proteins, under a wide range of conditions such as at elevated temperatures, for example, during food processing. This chapter describes the development of a model system to establish the extent of the early stage of this reaction in food proteins, under a variety of conditions.

4.2 The reaction of dehydroascorbic acid with a variety of proteins

To assess the generality of the crosslinking mechanism reported in lens proteins, the reactions of a number of different proteins with DHA were investigated. These included pepsin A, α-amylase, casein, ribonuclease A (RNAse A) and ovalbumin. Initial experiments, under model conditions, indicated that the reaction of DHA with pepsin A did not result in crosslink formation. The analysis of this reaction proved difficult, however, as self-digestion of the protein occurred under the reaction conditions. α-Amylase also proved difficult to study, although it appeared that protein aggregation, through crosslink formation, was occurring.

The reactions of ovalbumin and RNAse A with DHA resulted in the rapid formation of large protein aggregates. When ovalbumin was the protein component, numerous products of lower molecular weight were also produced. These compounds may result from the reaction of ovalbumin with DHA or oxygen radicals.[11] Examples of such radicals include those produced during the reduction of DHA,[12] or from the reaction of DHA with amino groups, as discussed in *chapter three.*

The majority of the proteins tested appeared to undergo aggregation, indicating that the reaction is a general one. Having surveyed several proteins, RNAse A was selected as the protein component for our model reactions. RNAse A is readily available in a purified form, and is heat stable, allowing the study of chemical derivatisation over a wide range of temperatures. Also, as it is a small protein (13680 Da), oligomerisation of protein monomers was easily visualised.

RNAse A contains eleven free amino groups, corresponding to the ε-amino groups of the ten lysine residues, and the single terminal α-amino group.[13] Due to the hydrophilic nature of the ε-amino group, each of the ten lysine residues can be found on the surface of the folded protein, as shown in *figure 4.1.*

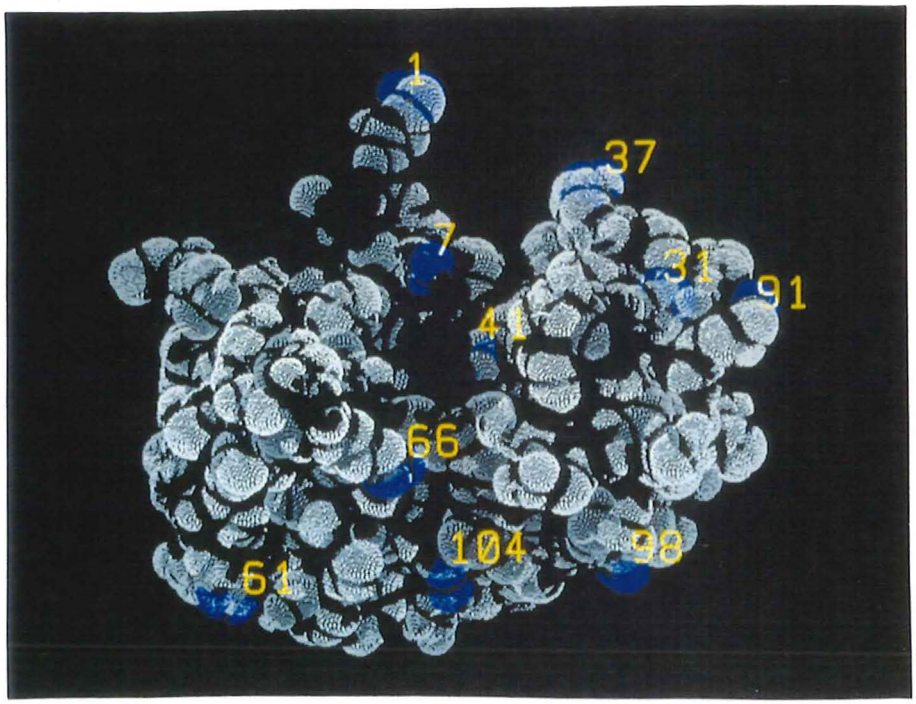


Figure 4.1: the protein RNAse A with its eleven amino groups highlighted in blue.[14]

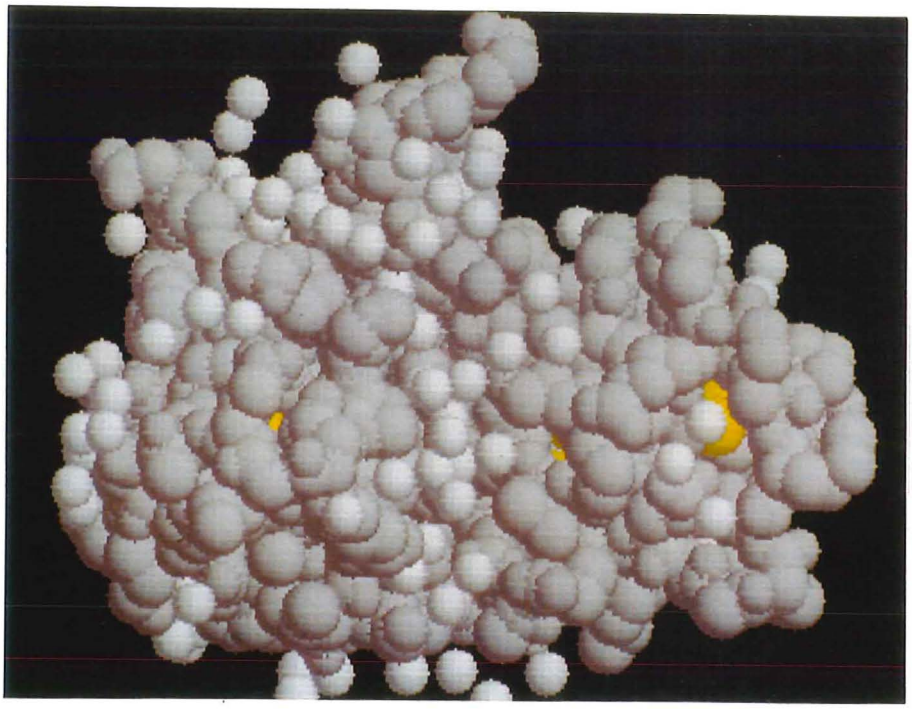


Figure 4.2: the protein RNAse A with its eight cysteine residues highlighted in yellow.[15]

The protein also contains eight cysteine residues, generally found as four disulfide bonds in the centre of the protein molecule, as shown in *figure 4.2.*

As was discussed in *chapter three*, the hydrophobicity of the cysteine residues results in their reduced availability for crosslinking reactions, as they are buried in the centre of the folded protein. This is in contrast to the hydrophilic lysine residues which are readily available for reaction.

4.3 Initial comparison of the Maillard reactivity of dehydroascorbic acid with ribonuclease A under a range of conditions

Three concentrations of protein were investigated: 10 mg/mL, 25 mg/mL and 50 mg/mL. Each concentration of protein was incubated, with DHA, at two different temperatures, either 37°C or 50°C. In addition, a control was run, identical for each experiment, but with DHA omitted. Proteins incubated with DHA at higher temperatures showed rapid aggregation, but were difficult to analyse. Lower temperatures were, therefore, studied in the first instance.

Visual inspection of the products of the reaction of DHA with RNAse A, after incubation, showed a rapid increase in the presence of coloured products. In *figure 4.3*, it can be seen that four tubes, on the right of the figure, containing 25 mg/mL of both RNAse A and DHA, have become highly coloured after being incubated at 37°C. The control tube, however, has remained colourless. This indicates that Maillard-type chemistry is occurring, *via* the formation of a coloured Schiff base. Disulfide bonding may also be occurring, but does not account for the observed colour change.

The Maillard reactivity of the reaction was, therefore, examined using similar methodology to that used for the amino acid model systems described in *chapter three.*

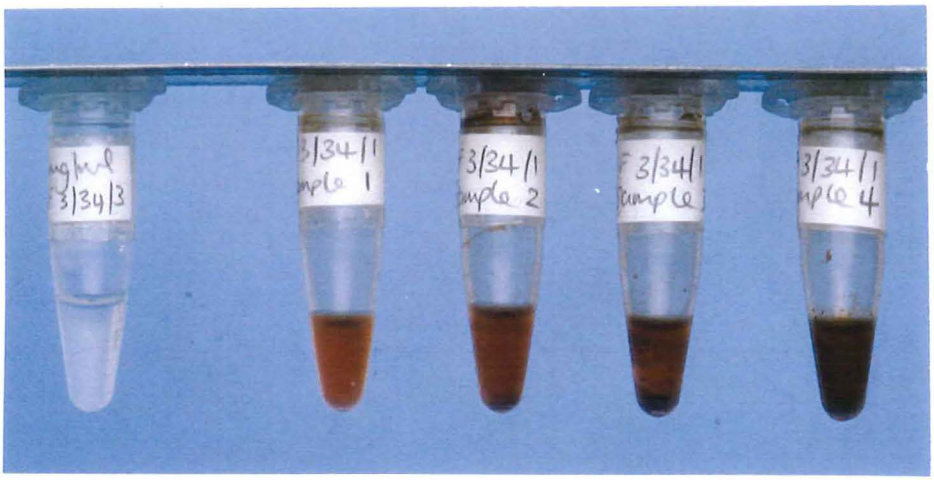


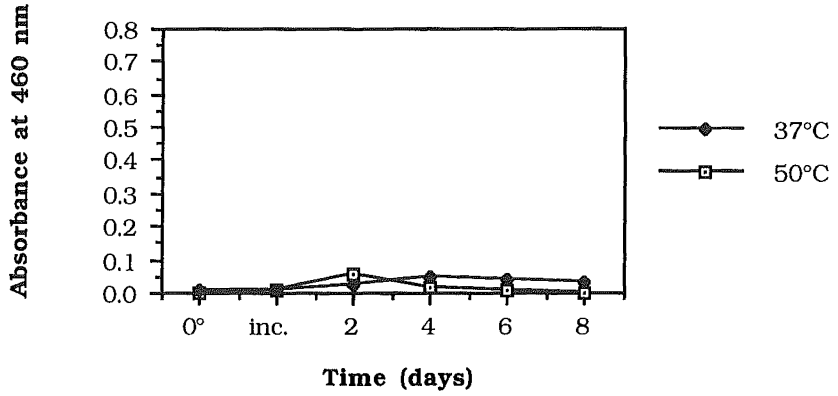
Figure 4.3: increased formation of coloured products with increasing time. Note the formation of insoluble protein aggregate from day six.

When compared to the amino acid-DHA model systems discussed in *chapter three*, the increase in absorbance over time, shown in *figure 4.4*, is not inconsistent with the occurrence of similar Maillard chemistry. It can be seen that increasing the concentration of RNAse A results in an increase in the rate of the browning reaction. Peak browning appears to have occurred on day two, for all but one of the reaction systems, which is then followed by a decrease in absorbance. We attribute this to the formation of brown coloured, insoluble aggregated material, which can be seen in *figure 4.3*. The mass of this insoluble material was found to increase with both increasing time and concentration.

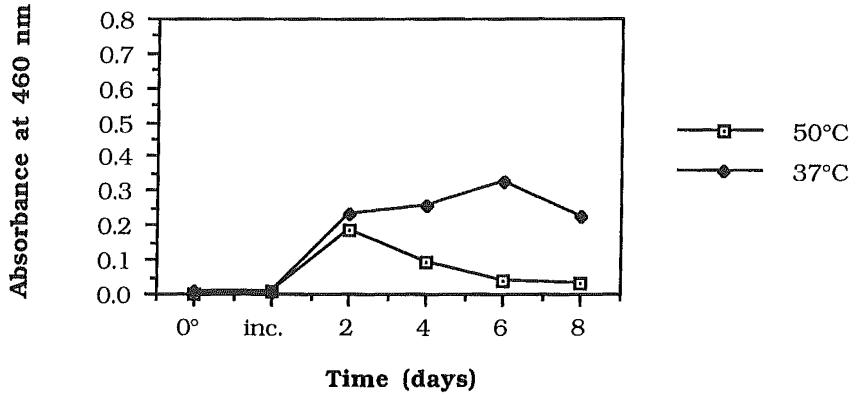
The formation of this precipitate may also explain the difference in absorbance between the 37°C samples and those of the 50°C samples. From the graphs, it appears that the rate of the browning reaction is greater at the lower temperature. However, the mass of precipitate produced at 50°C is generally greater than that of the equivalent 37°C sample. It is most likely, therefore, that the rate of reaction is greatest at the higher temperature, where the majority of the protein has formed aggregated material within the first four days of reaction.

Therefore, although the increase in colour, measured by its absorbance at 460 nm, indicates that Maillard chemistry is occurring, it does not provide a useful measure of the rate of protein aggregation.

a) reactions of DHA with RNAse A at a protein concentration of 10 mg/mL



b) reactions of DHA with RNAse A at a protein concentration of 25 mg/mL



c) reactions of DHA with RNAse A at a protein concentration of 50 mg/mL

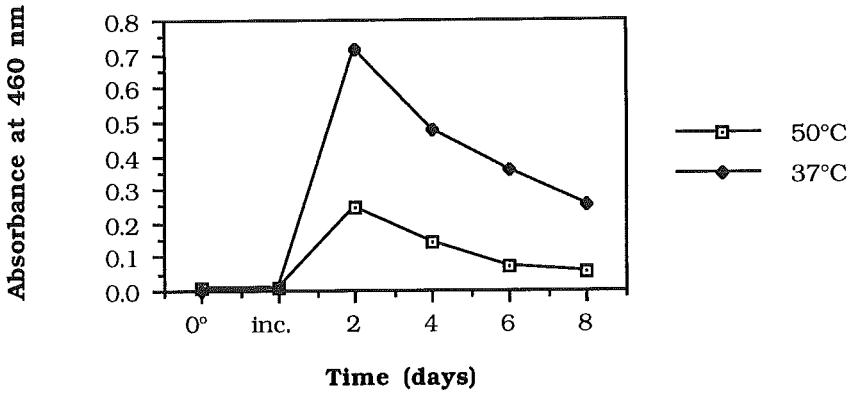


Figure 4.4: comparison of the Maillard reactivity of DHA with RNAse A when incubated at either 37°C or 50°C, at a variety of concentrations.

We propose that the formation of the insoluble aggregated material is, at least in part, a result of non-disulfide covalent crosslinking of protein monomers and that the reaction proceeds *via* the Maillard reaction at the ε-amino groups of lysine residues. Which molecules are directly responsible for crosslink formation, and the mechanism by which this occurs, requires investigation.

4.4 *Comparison of the crosslinking reaction under a range of conditions*

An electrophoretic method was sought which could provide information about DHA-mediated crosslinking. Methods for electrophoretic separation can be divided into two classes: separation by mass and separation by charge.

4.4.1 *Urea-polyacrylamide gel electrophoresis method for the analysis of dehydroascorbic acid-protein systems*

In the first instance, a polyacrylamide gel electrophoresis (PAGE) technique, which separated proteins by their differences in charge, was used.[13] Any modification in the charge of the overall protein will alter the mobility of that protein through the urea-PAGE gel,

making it possible to look at changes in specific amino acid residues. Since we have proposed that protein crosslinking occurs at the amino groups of lysine residues, this mechanistic study focussed on the affect of DHA on these residues.

Reaction of amino groups with succinic anhydride converts them from basic to acidic groups, resulting in a change in the net charge of the protein, by up to two unit charges. This reaction (shown later in *figure 4.10*) and the conditions for the complete acylation of RNAse A established here, proved useful for further mechanistic studies, described later in this chapter.

Gradual succinylation of amino groups can be accomplished simply by adding increasing amounts of reagent, until all amino groups are modified. These species can then be separated by urea-PAGE electrophoresis, where the ionic charge affects the mobility of each species. This procedure can then be repeated using RNAse A which has been reacted with DHA, thus enabling a comparison to be made between the DHA-protein system and that of the unreacted protein.

After modifying RNAse A, it was electrophoresed on a homogeneous PAGE gel which contained 11% acrylamide, 0.07% bis-acrylamide and 8M urea, as a denaturing agent.

A number of difficulties were encountered in the optimisation of this technique, one of which was intense over-heating at the solvent front. This affected the quality of the separation and the reproducibility of the gel. A number of attempts were made to minimise these problems and, although differences in mobility upon modification were clearly demonstrated, the reproducibility of the gel was not sufficient to derive reliable mechanistic information.

4.4.2 *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis method for the analysis of dehydroascorbic acid-protein reaction systems*

To investigate the formation of non-disulfide crosslinks, it was necessary to develop a technique which enabled the separation and identification of any crosslinked products. Intermolecular crosslinking of proteins is readily detected since this process results in multimeric protein, with a consequent change in mass. This change in mass can be observed after an electrophoretic protocol in which separation is based on differences in mass. Electrophoresis in a reductive environment ensures that all disulfide bonds are broken.[16] Changes in mass detected under these conditions must, therefore, be attributed to alternative crosslinking methods, such as those resulting from reaction at the ε-amino group of lysine residues.

A polyacrylamide gel electrophoresis method was used, which contained the negatively charged detergent sodium dodecyl sulfate (SDS). SDS acts as a denaturing agent and also confers a uniform negative charge density upon the protein-detergent complexes. As a result, the proteins migrate through the gel matrix at a rate determined by their molecular size, rather than by the charge conferred by their amino acid composition.

The gel conditions were optimised to enable the visualisation of large molecular weight protein aggregates. The gel porosity of the resolving gel was varied initially so that migration of monomeric RNAse A to the bottom of the gel could be achieved on a reasonable timescale. The optimum acrylamide and bis-acrylamide concentrations for this to occur were found to be 12.2% and 0.33% respectively. A discontinuous electrophoresis system was used so that sharp bands of protein were produced in the electrophoresed gel. The stacking gel contained 3.5% acryamide and 0.1% bis-acrylamide.

Having optimised the gel conditions for the protein RNAse A, it was necessary to manipulate the volume of protein loaded on the gel, for the different concentrations which were to be investigated. The

optimum load volumes were found to equate to 250 µg of protein in each case. The large volumes of protein loaded on the gel resulted in overloaded monomeric protein, but were necessary for the detection of oligomers formed early in the reaction.

The above conditions were also found to be suitable for the analysis of a range of other proteins used in this investigation.

4.4.3 *Effect of crosslinking agent and reaction time on the rate of the crosslinking reaction*

Having optimised the gel conditions, control gels of protein-only samples, which had been incubated at either 37°C or 50°C for eight days, were analysed. An example of these gels can be seen in *figure 4.5.*

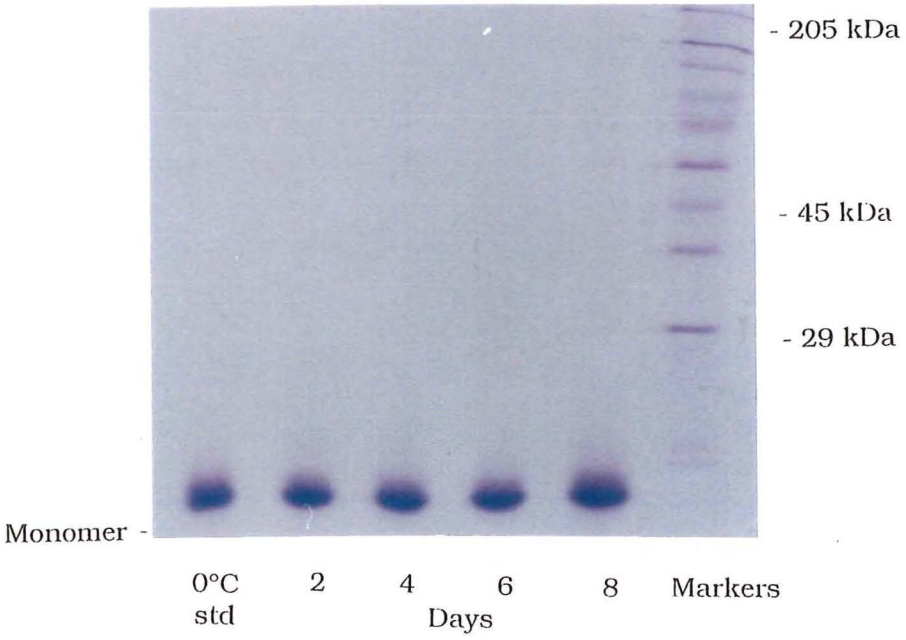


Figure 4.5: SDS-PAGE gel of RNAse A incubated at 37°C for a period of eight days, showing the presence of monomeric RNAse A.

Some batches of RNAse A used for this series of investigations, contained both dimeric and monomeric protein, as can be seen in *figure 4.6.* This dimeric protein was found in most of the batches

of commercial RNAse A, to varying extents, and have been noted in the literature.[17,18]

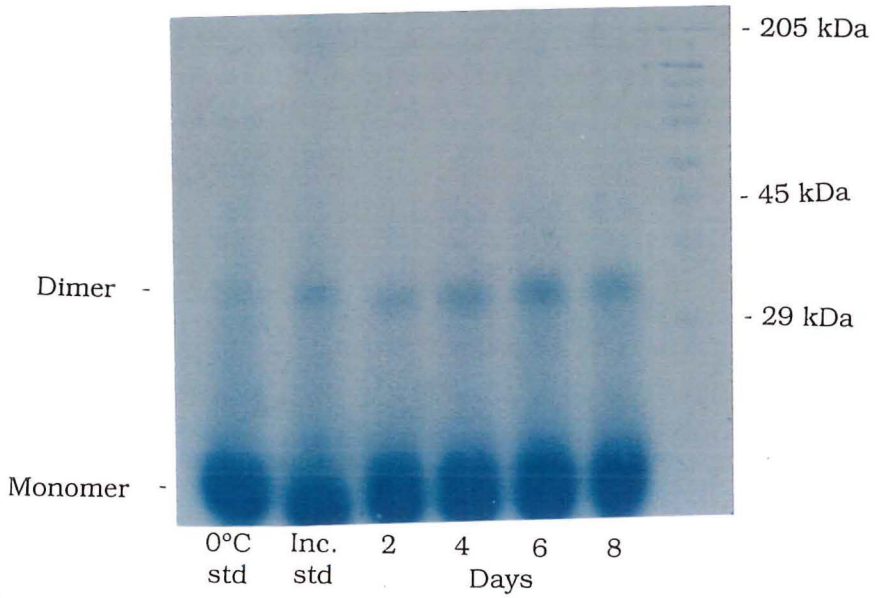


Figure 4.6: SDS-PAGE gel of RNAse A incubated at 37°C for a period of eight days, showing the presence of both monomeric and dimeric RNAse A.

When RNAse A was incubated with DHA under the same conditions, the RNAse A was found to covalently crosslink, resulting in considerably increased dimer formation and the formation of multimeric protein units, not observed in the control.

Figure 4.7 unambiguously demonstrates the formation of crosslinked protein, with increasing time. Since the gel has been run under reducing conditions, which leads to the cleavage of any disulfide bonds present, crosslinking must be a result of a process other than disulfide bonding.

Large protein aggregates were found to form as early as day two of the reaction, continuing to increase over the duration of the experiment. By day eight, material much greater than 205 kDa, the largest of the molecular weight standards, could be seen at the top of the gel. As RNAse A has a molecular weight of only 13.7 kDa, this represents highly crosslinked protein. As described previously,

each of the samples contained insoluble aggregated material which was unable to enter the gel. It would be reasonable to assume, therefore, that material of very much greater mass than that shown on the gel is also being formed, and that we are only observing the early stages of the aggregation process.

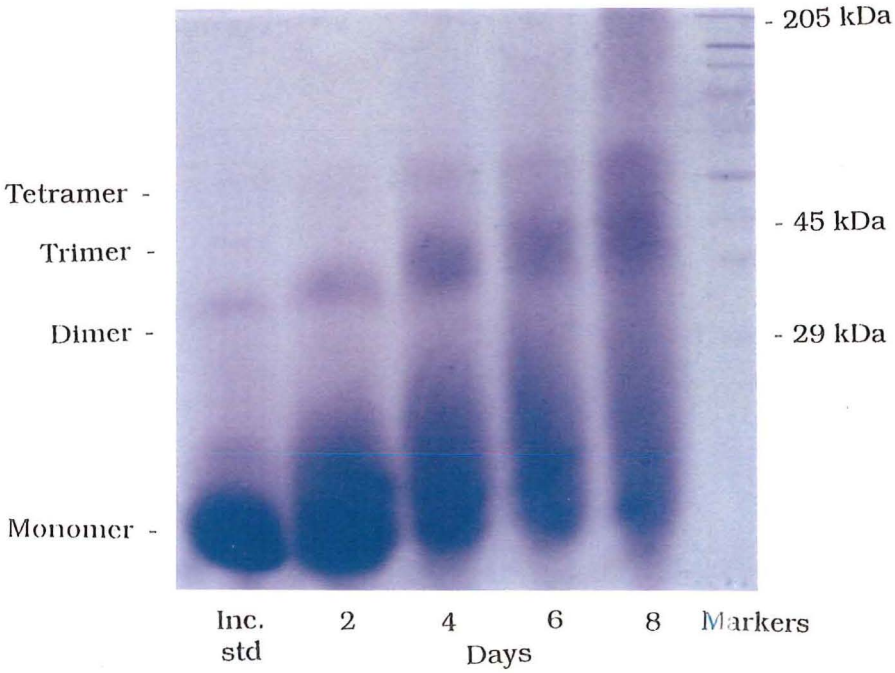


Figure 4.7: SDS-PAGE gel of the reaction between RNAse A and DHA at a concentration of 25 mg/mL and an incubation temperature of 37°C.

When this SDS-PAGE gel was compared with that of either ascorbic acid or xylose with RNAse A, it was found that DHA is far more reactive under these conditions. When ascorbic acid was reacted with RNAse A, no crosslinking was observed, confirming that DHA is essential for the reaction. The reaction of xylose with RNAse A produced a similar crosslinking pattern to DHA, although at a slightly slower rate than that of DHA. These results are consistent with the results of the DHA-amino acid model system discussed in *chapter three.*

4.4.4 *Effect of protein concentration and incubation temperature on the crosslinking reaction*

The effect of temperature and concentration on the crosslinking reaction were investigated. Each of the reaction systems described

previously were analysed by SDS-PAGE and, in each case, a similar crosslinking pattern resulted. The concentrations of the multimeric protein units, as judged by the intensity of the bands, were shown to increase with increasing concentration and temperature.

As conditions approached those relevant to food processing, that is higher incubation temperatures and protein concentrations, the formation of insoluble aggregates proceeded to such an extent that no protein remained in solution. In these cases, no protein bands were visualised on the electrophoretic gel. Reduction of this insoluble material, with mercaptoethanol, did not increase the solubility of the material, indicating that protein aggregation was not entirely a result of disulfide bonding. This suggests that non-disulfide crosslinking is likely to be important in food processing, but as the large aggregated protein is unable to enter the gel, the extent of this reaction is hard to measure using this methodology.

The formation of protein crosslinks was most easily observed at a protein concentration of 25 mg/mL and at an incubation temperature of 37°C. Also, under these conditions, the formation of insoluble aggregates did not appear to affect the intensity of the protein bands on the electrophoretic gel. These conditions were, therefore, selected as the model reaction conditions for mechanistic investigations of the early stages of the aggregation process.

4.4.5 Effect of pH on the rate of the crosslinking reaction

As pH has an important influence on both the rate of reaction, and the types of products formed, Maillard reaction systems are often studied with and without pH control, particularly in the study of low molecular weight coloured compounds.[19] Both conditions may be relevant in food systems as, although some systems are buffered, in others the pH may vary during processing.

The initial reaction systems had been carried out in aqueous solutions with no pH control. However, when the pH was monitored over the course of the reaction, it was found that the

protein standard solution had a stable pH of 7. After addition of DHA, the pH of the solution immediately drops to approximately 4 and continued to drop as the reaction proceeded, presumably due to the release of acidic by-products.

Therefore, the reaction of DHA with RNAse A, with the pH controlled, was also investigated. This was achieved by dissolving RNAse A, both with and without DHA, in a selection of buffer solutions. These included borax buffer (pH 9.1), phosphate buffer (pH 6.8) and phthalate buffer (pH 4.0).

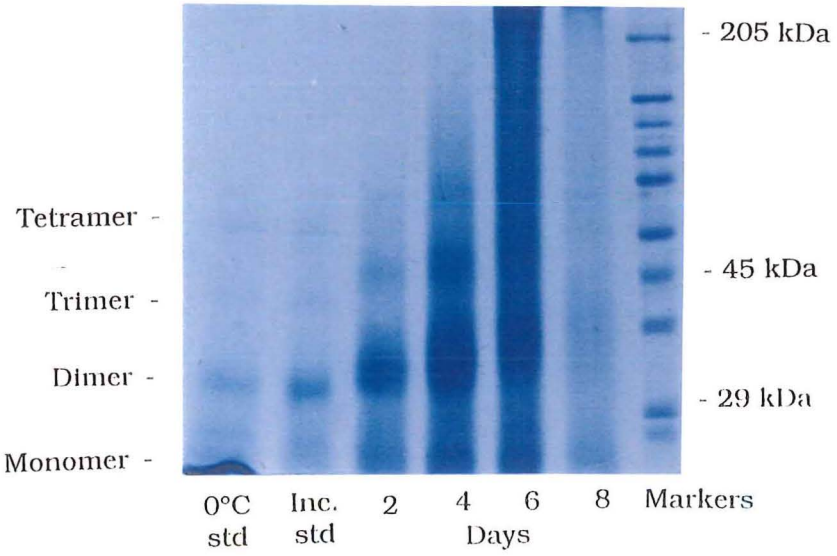


Figure 4.8: SDS-PAGE gel of the reaction between RNAse A and DHA at a concentration of 25 mg/mL and an incubation temperature of 37°C in phosphate buffer (pH 6.8).

At both pH 4.0 and pH 6.8 a similar crosslinking pattern to that of the uncontrolled systems was shown. In the more acidic pH, however, the rate of the browning reaction had increased. This was demonstrated by the immediate formation of large quantities of brown, insoluble protein aggregates. The reaction system at pH 6.8, shown in *figure 4.8*, produced much smaller quantities of precipitate which were only apparent on days six and eight. The rate of browning, and the quantity of precipitate, of this system was very similar to that of the reaction where the pH was not controlled. At each pH studied, the control solution, containing RNAse A only, remained colourless, indicating that the browning

reaction is not merely a function of pH but requires the presence of DHA.

A different range of products appear to be formed in alkaline solutions. In this case, no browning or precipitation was observed. The SDS-PAGE gel of this system indicated a decrease in the rate of the crosslinking reaction as well as a decrease in the number of products formed, as shown in *figure 4.9.*

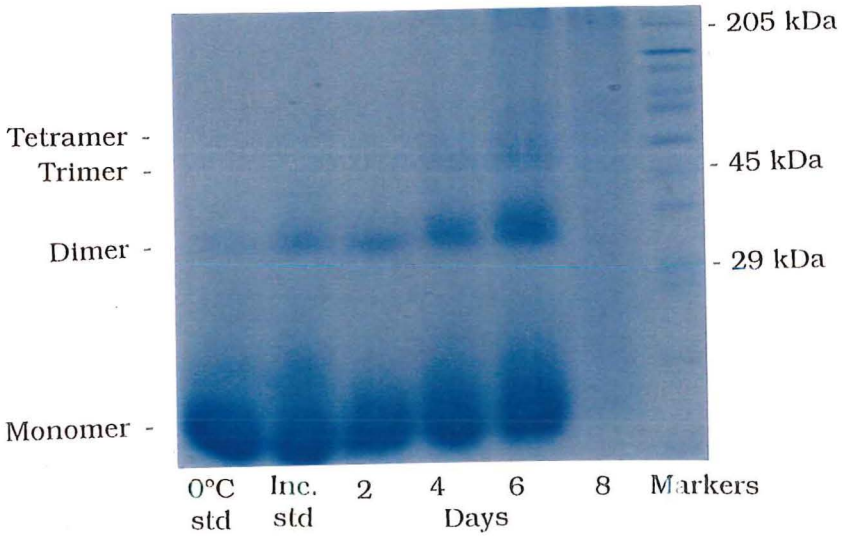


Figure 4.9: SDS-PAGE gel of the reaction between RNAse A and DHA at a concentration of 25 mg/mL and an incubation temperature of 37°C in borax buffer (pH 9.1).

The rates of both the browning reactions and the protein crosslinking reaction, therefore, seem to be favoured in the more acidic solutions, with the reactions proceeding further when compared with the more alkaline system. This effect is in contrast to that observed in the study of sugar-amino acid systems. When the absorbance of the reaction system is monitored at a single wavelength, in the range 420-490 nm, it is reported to increase over the pH range 4-8, with a maximum absorbance occurring at pH 10.[19] This reaction is probably faster in base as the amino group will be in a deprotonated form and, therefore, more reactive. Although this also applies in the DHA-amino acid reaction systems, the instability of DHA in alkaline solutions, where it is rapidly

hydrolysed to give 2,3-DKG,[20] is likely to greatly decrease the overall rate of the reaction. This may suggest that the crosslinking of protein is due to DHA itself and not the alkaline hydrolysis products.

4.5 *Investigation into the mechanism of the protein crosslinking reaction*

Our preliminary evidence had suggested that the amino acid lysine is involved in protein crosslinking *via* Maillard chemistry. In order to corroborate this theory, RNAse A was selectively protected at both the cysteine residue and the lysine residue, prior to reaction with DHA.

4.5.1 *Reaction of dehydroascorbic acid with ribonuclease A containing capped lysine residues*

If our theory was correct, then we predicted that acylation of the ε-amino group of the lysine residues should prevent the observed formation of multimeric protein aggregates.

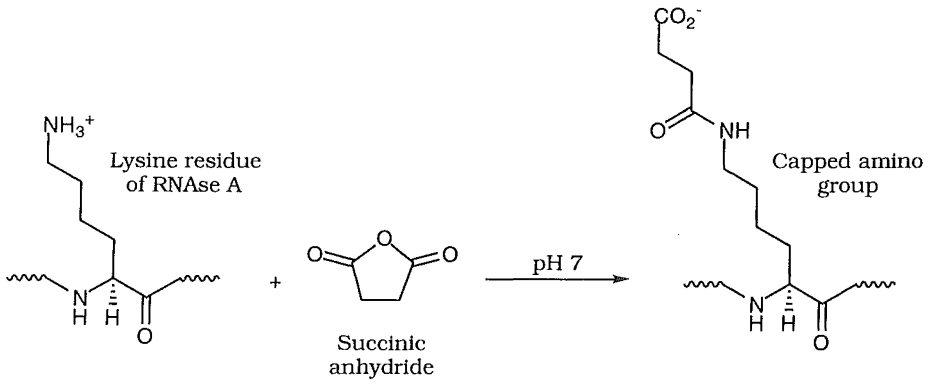


Figure 4.10: capping of lysine residues with succinic anhydride.

The eleven free amino groups of RNAse A were, therefore, selectively protected by reaction with succinic anhydride under conditions established previously (see section 4.4.1), which gave complete acylation (*figure 4.10*).[13]

Having capped all of the amino groups of the protein, it was incubated with DHA under model conditions. The SDS-PAGE gel of this reaction, shown in *figure 4.11*, showed that no DHA-mediated non-disulfide crosslinking has occurred, in contrast to *figure 4.7*, which showed extensive crosslinking. This provides compelling evidence that the formation of covalent crosslinks between protein monomers, demonstrated previously, has occurred *via* reaction at the lysine residue.

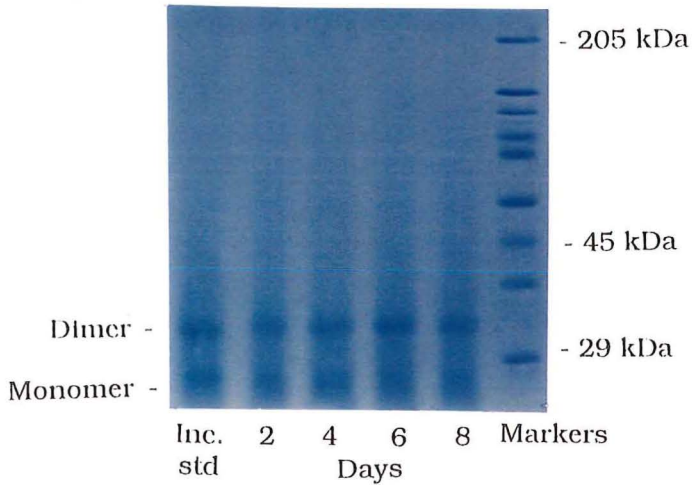


Figure 4.11: SDS-PAGE gel of the reaction between derivatised RNAse A, with capped lysine residues, and DHA at a concentration of 25 mg/mL and an incubation temperature of 37°C, without pH control.

4.5.2 *Reaction of dehydroascorbic acid with ribonuclease A containing capped cysteine residues*

To further clarify that the reductive conditions of the SDS-PAGE gels were resulting in the cleavage of any disulfide bonds present, DHA was reacted with RNAse A in which the thiol groups were unavailable for reaction.

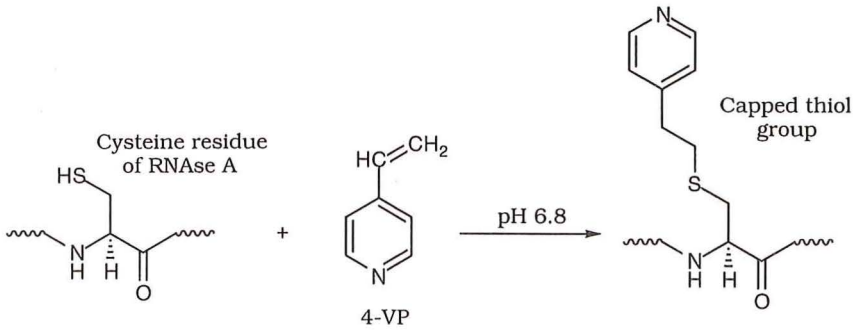


Figure 4.12: capping of cysteine residues with 4-VP.

The eight cysteine residues of RNAse A were selectively capped with 4-vinyl pyridine (4-VP), in the presence of the denaturing agent urea, and the reducing agent dithiothreitol (DTT) (*figure 4.12*).[21] Removal of the urea and the DTT was achieved by dialysis of the resulting solution.

The modified protein was incubated with DHA, in distilled water, at 37°C and with a protein concentration of approximately 25 mg/mL (assuming a 100% yield from the reaction of RNAse A with 4-VP).

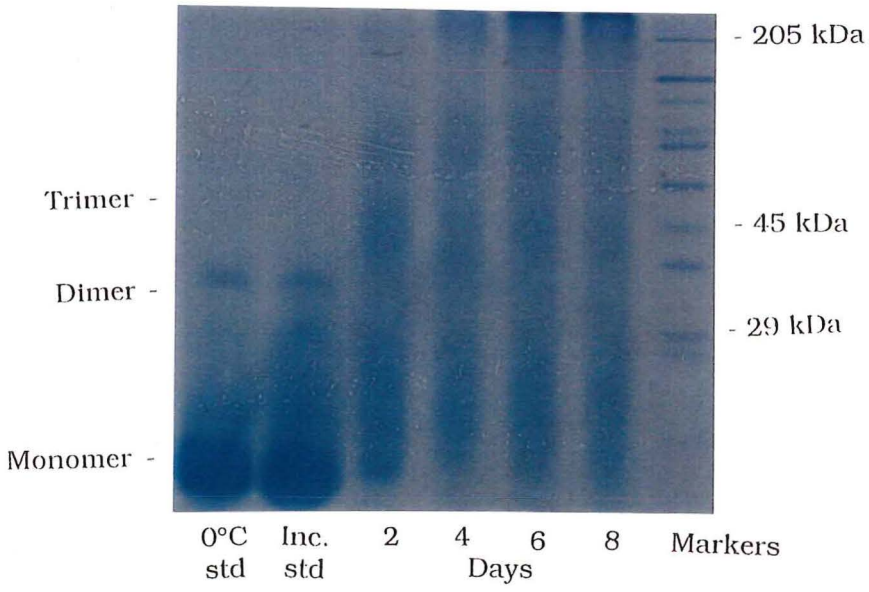


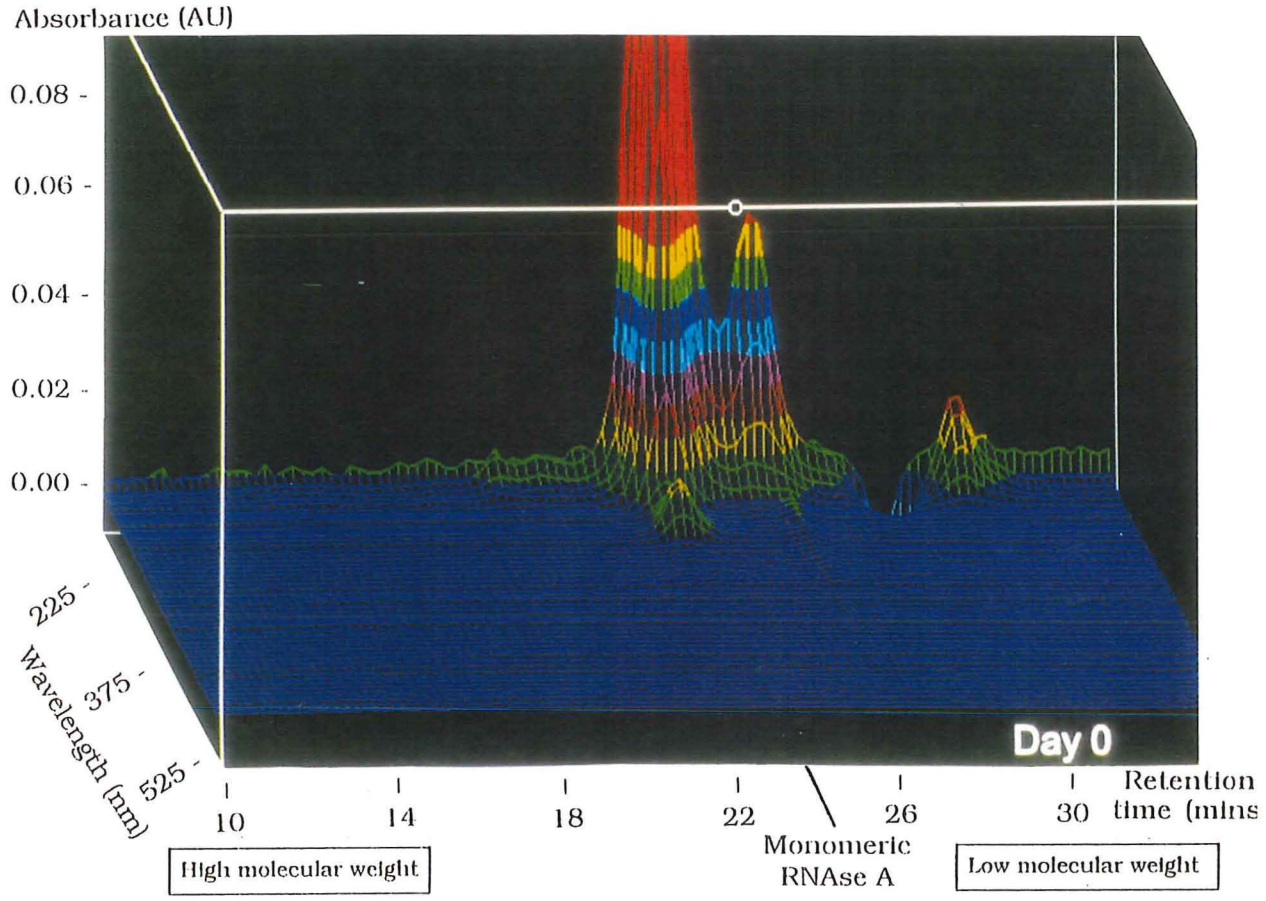
Figure 4.13: SDS-PAGE gel of the reaction between RNAse A, with capped cysteine residues, and DHA at a concentration of 25 mg/mL and an incubation temperature of 37°C, without pH control.

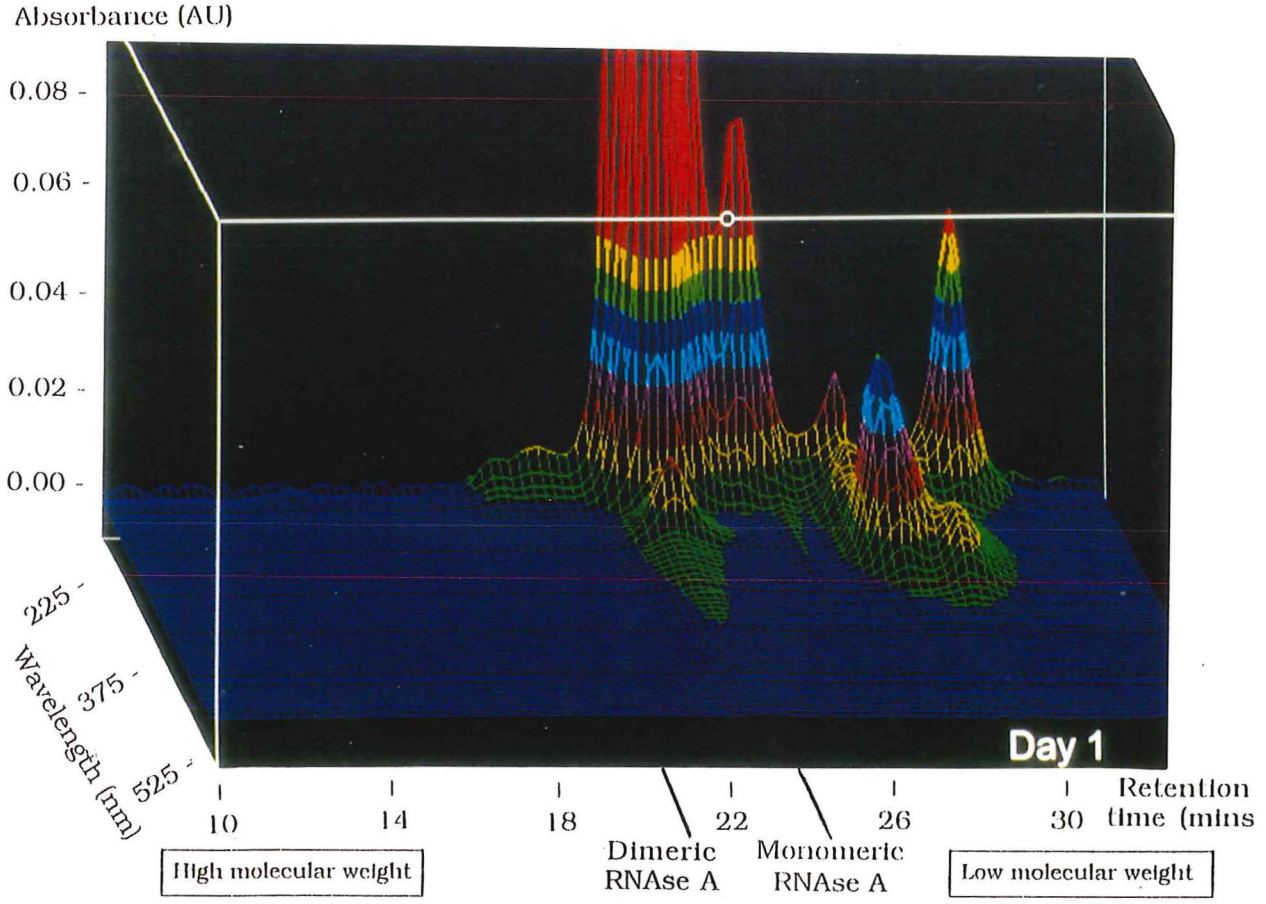
Visual inspection of the reaction samples indicated that the rate of the browning reaction was increased, compared with RNAse A containing free thiol groups. Large quantities of brown precipitate were present from day one of the reaction. Analysis by SDS-PAGE, under reducing conditions, confirmed that the degree of crosslinking had also increased on any given day (*figure 4.13*). This was demonstrated by the presence of protein aggregates, at the top of the stacking gel, from day two, which were too large to enter the gel. For uncapped protein (*figure 4.7*) this was not visible until day six.

It can be concluded, therefore, that disulfide bonding is not involved in the formation of the protein crosslinks visualised on the SDS-PAGE gel. It may also be possible to conclude that both disulfide and non-disulfide bonding are occurring in the reaction of DHA with the unmodified protein, and that this results in a decrease in the rate of reaction at lysine residues. This may be due either to a lowering in the concentration of DHA in the reaction mixture, which will be reduced to ascorbic acid, or the presence of disulfide bonding which may sterically hinder the formation of alternative covalent crosslinks.

4.6 *Analysis of the crosslinking reaction by size exclusion-high performance liquid chromatography*

We have proposed that the non-disulfide crosslinking, demonstrated by this series of reactions, is occurring through the formation of a Schiff base at the ε-amino group of lysine residues. We have strong corroborating evidence that reaction is, in fact, occurring at the lysine residue. Although the formation of coloured compounds may indicate that Schiff base formation is occurring, the mechanism of this reaction has not been determined. It has also not been possible to evaluate the rate at which crosslinking is occurring, since protein aggregates much greater then 205 kDa are unable to enter the electrophoretic resolving gel, so quantifying the amount of products formed was not trivial.


Absorbance (AU)
0.08
0.06
0.04
0.02
0.00
225
Wavelength (nm)
375
525
Day 0
10
14
18
22
26
30
Retention time (mins
Monomeric RNAse A
High molecular weight
Low molecular weight


Absorbance (AU)
0.08
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0.04
0.02
0.00
225
Wavelength (nm)
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Day 1
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14
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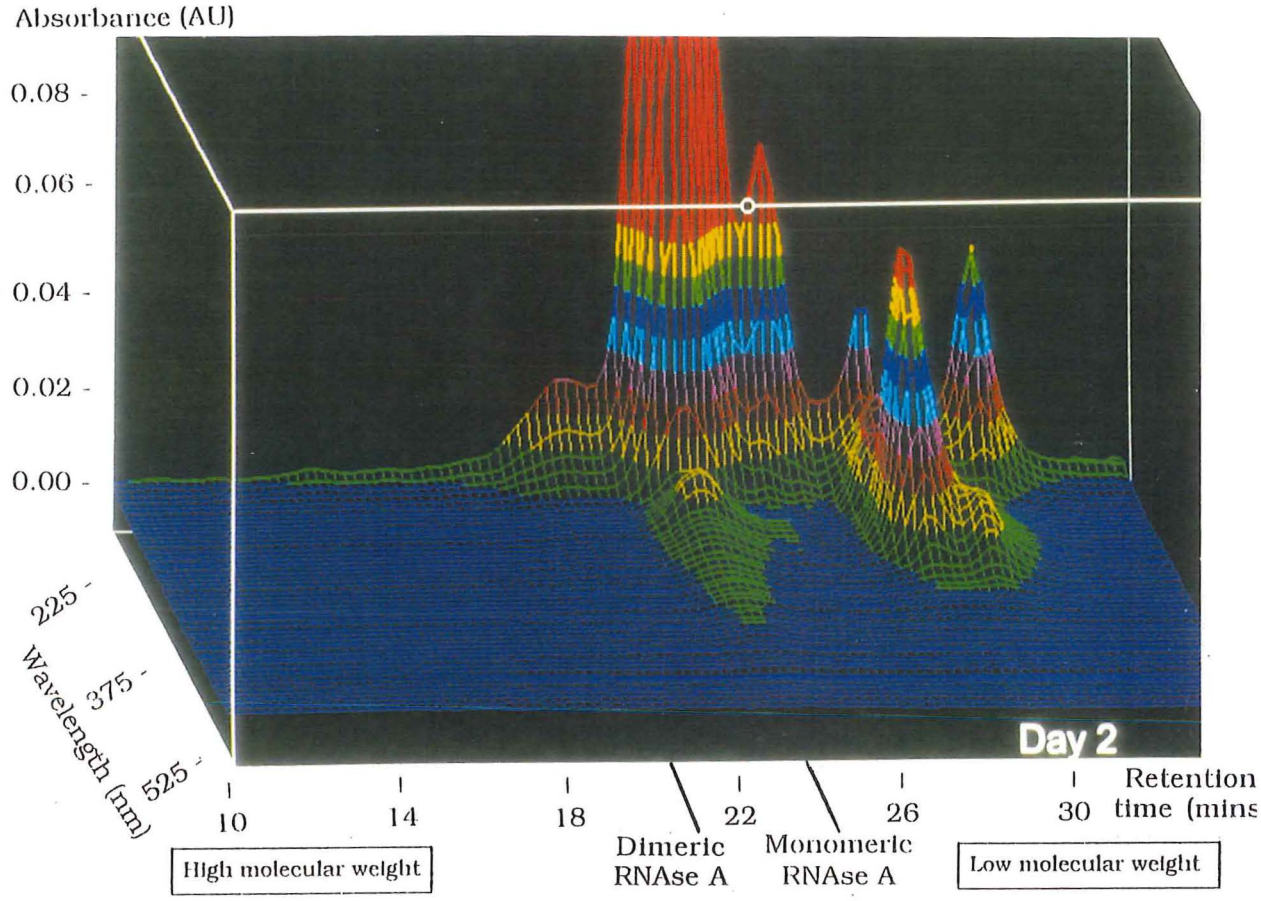
22
26
30
Retention time (mins
Dimeric RNAse A
Monomeric RNAse A
High molecular weight
Low molecular weight


Absorbance (AU)
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Wavelength (nm)
Day 2
10
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Retention time (mins
High molecular weight
Dimeric RNAse A
Monomeric RNAse A
Low molecular weight

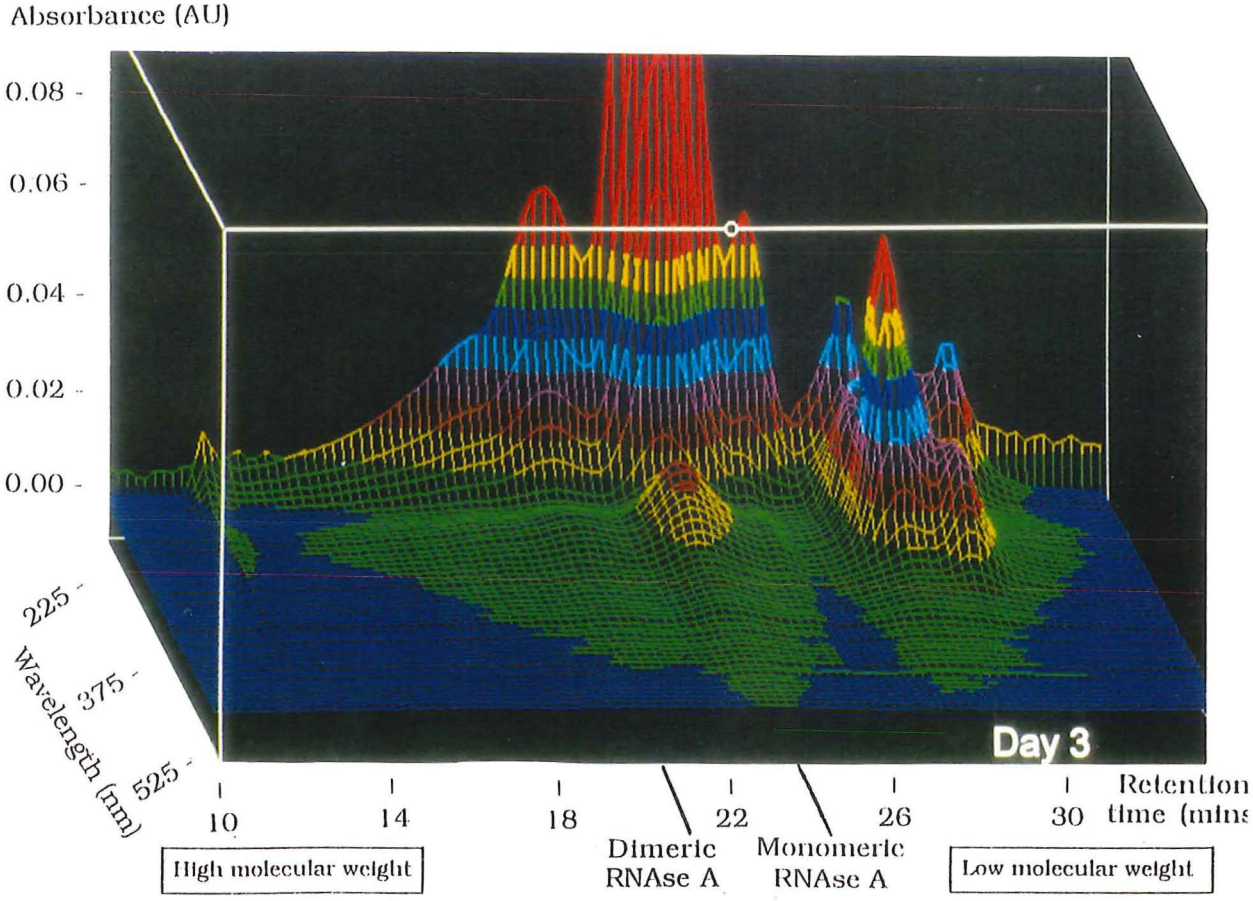

Absorbance (AU)
0.08
0.06
0.04
0.02
0.00
225
375
525
Wavelength (nm)
Day 3
10
14
18
22
26
30
Retention time (mins
High molecular weight
Dimeric RNAse A
Monomeric RNAse A
Low molecular weight

Figure 4.14: products of the reaction between DHA and RNAse A incubated at a concentration of 25 mg/mL and a temperature of 37°C followed by separation by SE-HPLC.

Therefore, in order to obtain further information about this reaction, fresh samples from a DHA-RNAse A reaction system were analysed by size exclusion-high performance liquid chromatography (SE-HPLC), with diode array detection.

DHA was incubated with RNAse A, under standard conditions. Aliquots were removed daily, immediately injected onto the SE-HPLC column and analysed under both reducing and non-reducing conditions. The three-dimensional chromatogram of the reduced samples showed that, as well as multimeric protein, compounds with lower molecular weights, compared with RNAse A, were present. The concentrations of these compounds increased with time, as shown in *figure 4.14*.

The formation of low molecular weight compounds may be due to radical chemistry involving DHA, as proposed for the reaction of DHA with ovalbumin.[11] These compounds, of lower molecular weight, may also be crosslinked, producing protein aggregates in a range of sizes. This may explain the 'smeared' appearance of the protein bands when analysed by SDS-PAGE. The three-dimensional chromatogram of the non-reduced samples showed the same range of products although there appeared to be a greater concentration of multimeric protein, compared to that of the reduced samples, presumably due to disulfide bonding.

The contour plots of the samples demonstrated a distinct shift of the protein envelope, into the visible region of the spectrum as shown in *figure 4.15*. This is consistent with Schiff base formation, an intermediate in our postulated crosslinking reaction, providing further evidence that reaction occurs at the ε-amino group of the lysine residue.

A shift in the protein envelope was only observed if the samples were analysed immediately after reaction. This is presumably due to the labile nature of the intermediate.

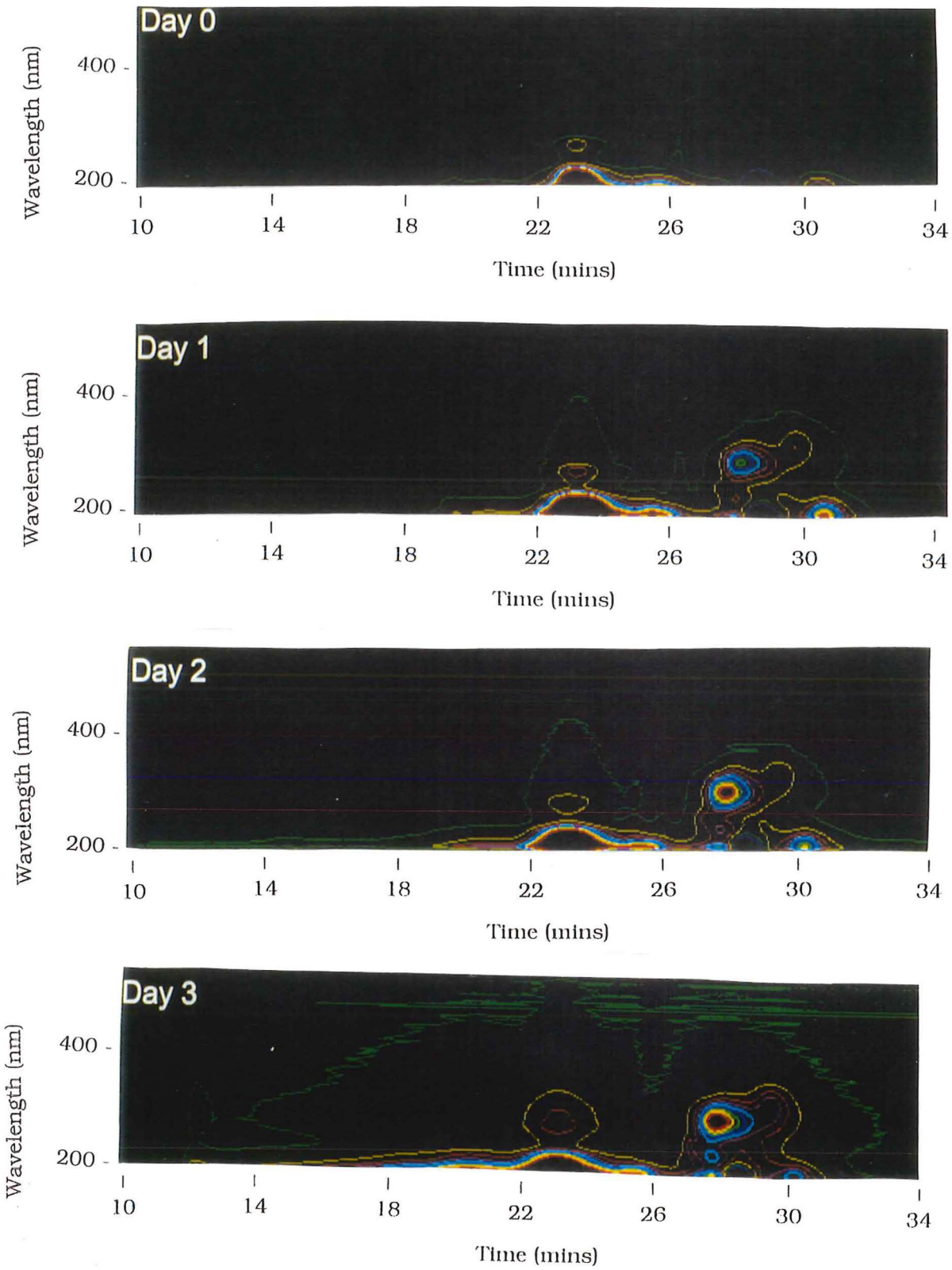


Figure 4.15: contour plots of the products of the reaction between DHA and RNAse A after separation by SE-HPLC.

Due to the intractable nature of the protein aggregate, measuring the rate of the reaction is a difficult task. SE-HPLC, however, allows an estimation of the rate of reaction, by monitoring the loss of protein monomer as the reaction proceeds.

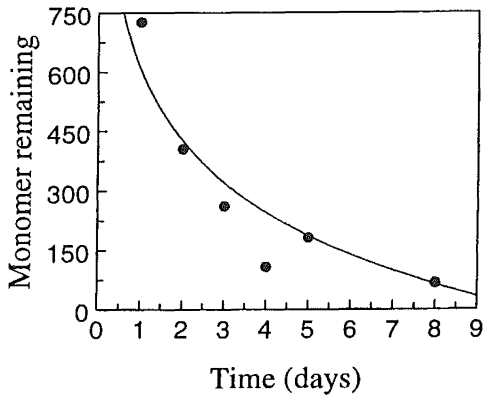


Figure 4.16: the decrease in concentration of monomeric RNAse A during the reaction of DHA with RNAse A, monitored by SE-HPLC.

In *figure 4.16*, the concentration of protein monomer can be seen to decrease exponentially, with the loss of half of the original protein concentration within the first two days of the reaction. The concentration continues to decrease, with less than 10% remaining after eight days. The rate of reaction, therefore, is rapid initially, but slows with the diminishing protein concentration. This corroborates earlier evidence that the mass of aggregated material was approximately equal to that of the total protein content by day eight of the reaction.

4.7 *Reaction of ribonuclease A with the degradation products of dehydroascorbic acid*

So far, this chapter has been concerned with the formation of protein crosslinks as a result of the addition of DHA to protein solutions. Under the reaction conditions, DHA can undergo a variety of reactions, resulting in the formation of a number of degradation products, including threose,[22] cyclotene,[23] and oxalic acid,[9] as was discussed in *chapter two*, which may also act as crosslinking agents with proteins.

The compound glyoxal, is believed to be an oxidation product of glucose,[24] and may be formed through the degradation of threose, an early breakdown product of glucose. As threose has been shown to be an oxidation product of DHA, we propose that glyoxal may also result from the degradation of DHA.

Each of the compounds shown in *figure 4.17*, may be capable of crosslinking proteins directly, and, therefore, could be involved in the crosslinking mechanism of DHA with proteins. To investigate this, they were incubated with RNAse A, under model reaction conditions and their crosslinking ability was analysed by SDS-PAGE.

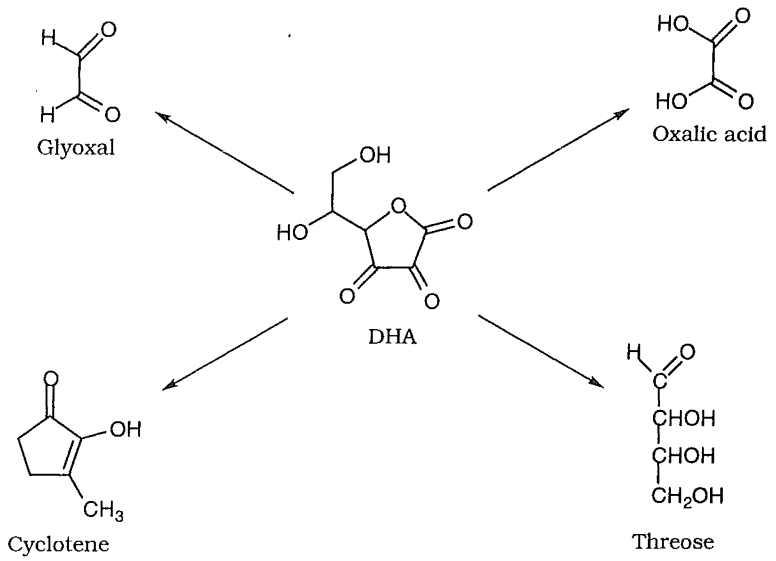


Figure 4.17: examples of degradation products of dehydroascorbic acid.

The reaction of RNAse A with oxalic acid (*figure 4.18*), did not result in the formation of protein crosslinks.

Figure 4.19 shows the products of the reaction of threose with RNAse A. Trimer formation is very rapid, occurring within the first few hours of the reaction and can be seen to increase with time. Multimeric protein is clearly visible on the second day of the reaction. Thus any threose produced from the degradation of DHA will be a very reactive crosslinking agent. However, our NMR study, in *chapter two*, suggested that the concentration of threose in the

reaction mixture is likely to be small. Similar results are observed when RNAse A is reacted with glyoxal.

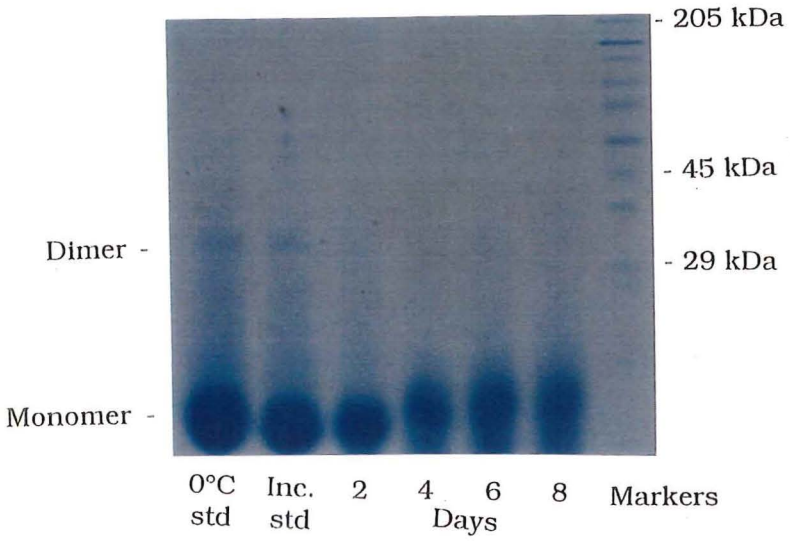


Figure 4.18: SDS-PAGE of the reaction between oxalic acid and RNAse A incubated at a concentration of 25 mg/mL and a temperature of 37°C.

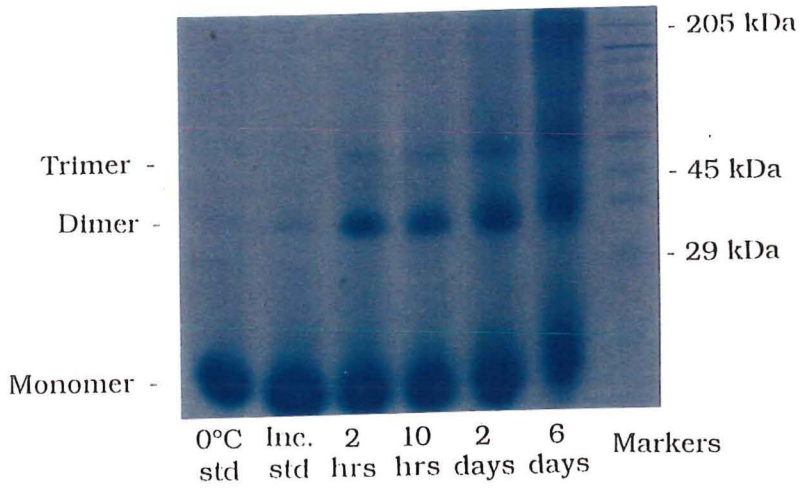


Figure 4.19: SDS-PAGE of the reaction between threose and RNAse A incubated at a concentration of 25 mg/mL and a temperature of 37°C.

When cyclotene is reacted with RNAse A under the same conditions, crosslinking of the RNAse A monomers is much slower, as shown in *figure 4.20.*

In this case, trimer formation can be seen on day six of the reaction, and shows a slight increase in concentration, as demonstrated by its increase in intensity, on day eight. Trimer concentration can, again, be seen to increase with time, although the rate of this reaction, compared with that of threose, is much slower. That is, the products formed in the reaction of RNAse A with cyclotene, over eight days, appear to be formed within hours of its reaction with threose.

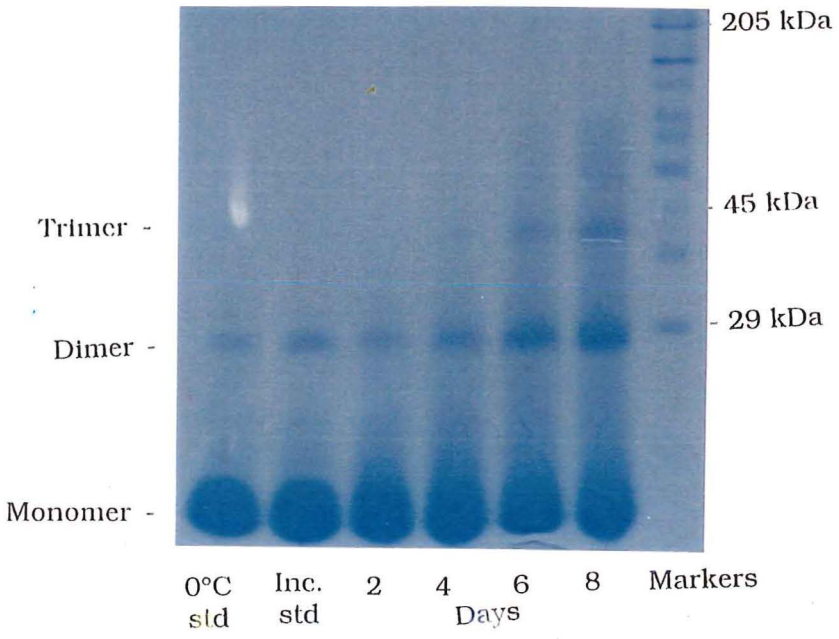


Figure 4.20: SDS-PAGE of the reaction between cyclotene and RNAse A incubated at a concentration of 25 mg/mL and a temperature of 37°C.

When the SDS-PAGE gels of DHA with RNAse A are compared with those of its degradation products, it can be seen that many more products are formed in the reaction of RNAse A with DHA directly. The electrophoretic gels of the reactions containing either cyclotene or threose, contain distinct bands, corresponding to dimer and trimer formation. Those involving DHA, however,

consistently contain bands which appear smeared. This may be due to the formation of protein subunits, *via* radical chemistry, which are subsequently crosslinked, resulting in products of many different sizes.

4.8 The reaction of ribonuclease A with cyclotene

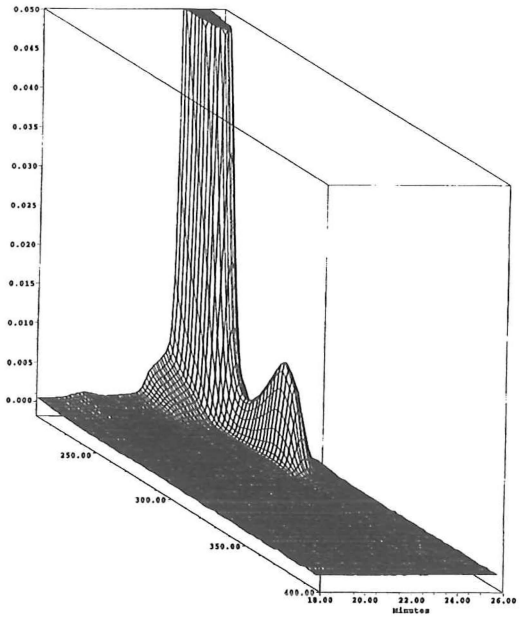
In order to obtain direct mechanistic information about the addition of DHA and its derivatives with proteins, samples were analysed by SE-HPLC. The RNAse A-cyclotene system were selected since they gave the cleanest results as judged by SDS-PAGE (*figure 4.20*). A control was also analysed which had been incubated under the same conditions, but with cyclotene omitted.

The absorption envelope of monomeric RNAse A, which has been incubated with cyclotene, can be seen to extend into the near UV, with increasing time, when compared with the control. As the change in the protein envelope is consistent with Schiff base formation, *via* the attachment of cyclotene to RNAse A, *figure 4.21* provides evidence for the formation of the crosslinking intermediate.

To further examine this reaction, the above samples were analysed by electrospray mass spectrometry.

Electrospray ionisation allows the production of intact ions *in vacuo,* from solubilised proteins, without degradation. Measuring the response of these ions to electric or magnetic fields results in a sequence of peaks whose component ions are multiply charged, the ions of each peak differing by one charge from those of adjacent neighbours in the sequence. Analysis of these peaks allows the determination of the ion to within a few atomic mass units.[25]

a) control sample containing RNAse A only showing the RNAse A absorption envelope at a retention time of 25 minutes



b) samples containing both RNAse A and cyclotene, incubated at 37°C for either four days or six days, showing the RNAse A absorption envelope extending into the near UV. Dimer formation can be seen at a retention time of 23 minutes.

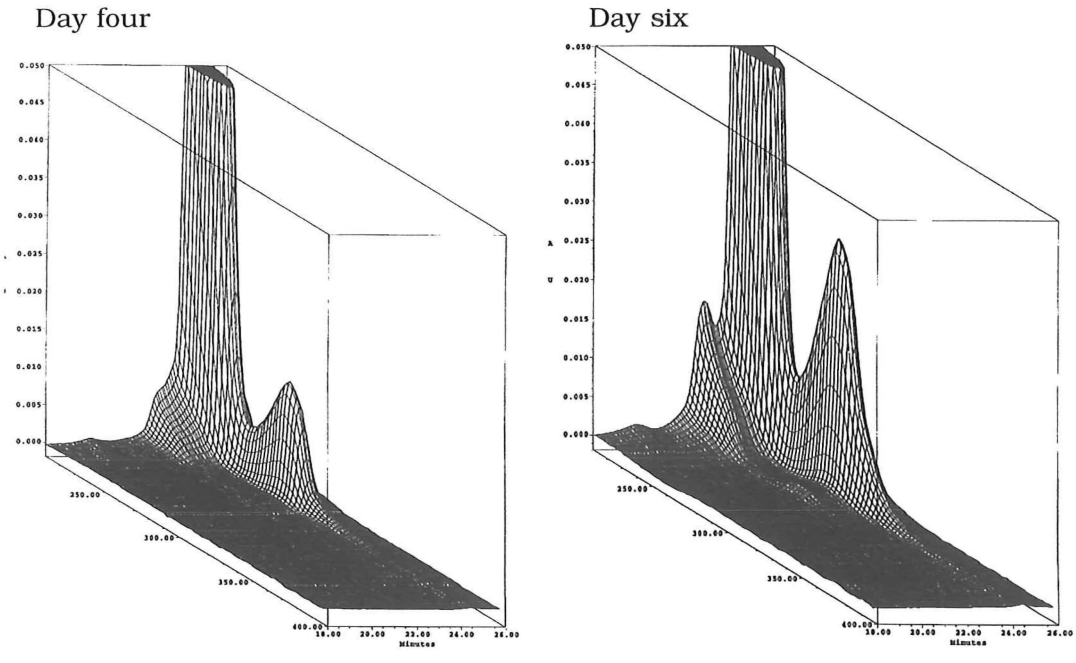
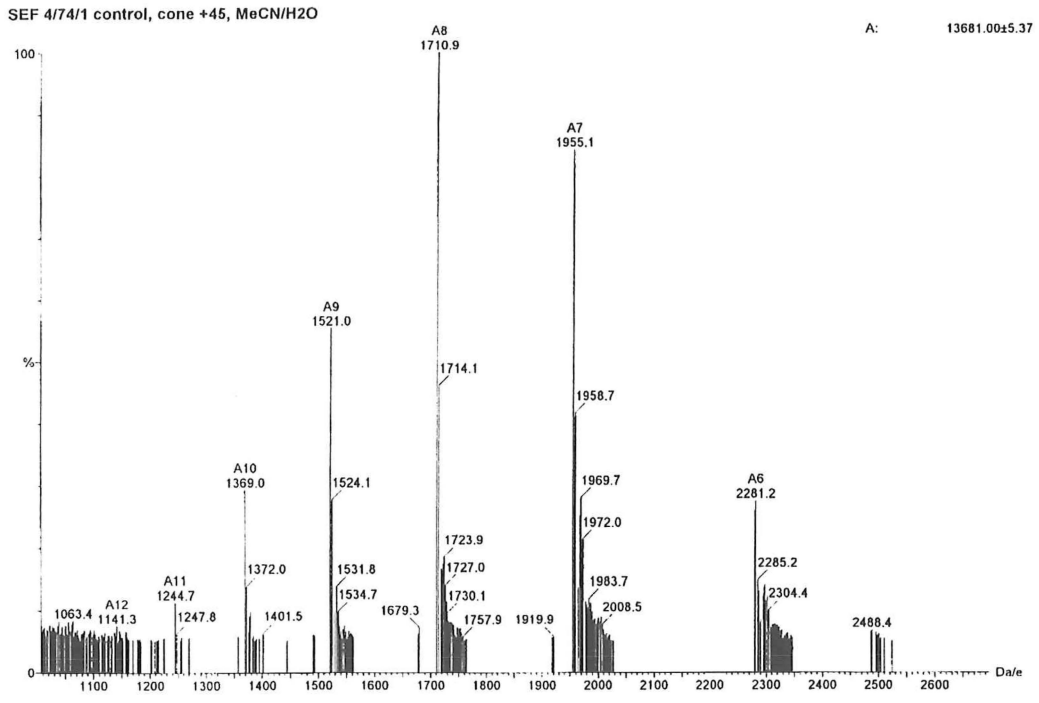


Figure 4.21: SE-HPLC chromatograms of an RNAse A control and the RNAse A-cyclotene reaction products after incubation at 37°C and a protein concentration of 25 mg/mL.

Analysis of the control experiment demonstrated the presence of one series of peaks with a mass of 13681±5.37, consistent with the literature mass of this protein, which is 13680 Da.[18] In the cyclotene-RNAse A samples, five series of peaks were found of mass weights of 13780.95±6.03, 13883.90±9.78, 13983.97±7.59 and 14092.22±14.00.

The first of these corresponds to unreacted protein, shown in *figure 4.22a.* The remaining four series of peaks, of decreasing intensity, are consistent with the successive addition of four molecules of cyclotene, of molecular mass 96 kDa, to the protein monomer (*figures 4.22b and 4.23*) with the overall loss of two water molecules.

a) control sample containing only RNAse A showing a single series of peaks consistent with the presence monomeric RNAse A



b) sample containing both RNAse A and cyclotene showing the four protein adducts with molecular weights consistent with the successive addition of four molecules of cyclotene and the loss of two water molecules

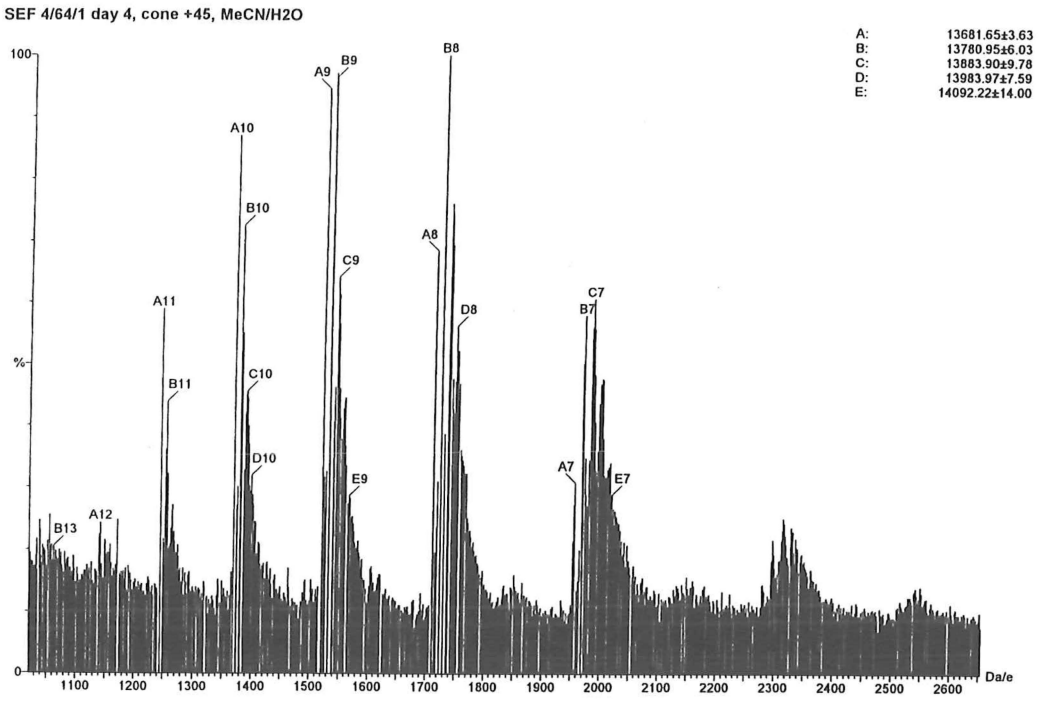


Figure 4.22: electrospray mass spectrometry spectra of the RNAse A-cyclotene reaction products after four days of incubation at 37°C at a protein concentration of 25 mg/mL.

We propose that these adducts are the intermediates of the observed crosslinking reaction. We also suggest that the resulting protein crosslinks are formed either by the formation of a Schiff base followed by a 1,4-addition reaction, or by cyclotene behaving as a dicarbonyl compound, as shown in *figure 4.24*. As cyclotene was previously shown to exist in the enol form,[26] it is likely that protein crosslinks result from the former reaction mechanism. An analogous mechanism can be postulated for DHA. This, however, requires further investigation.

Number of cylcotenes reacted	Calculated mass of observed protein adduct (A), assuming each forms a Schiff base	Calculated mass of observed monohydrate (A + 18)	Calculated mass of observed dihydrate (A + 36)	Recorded mass
0	**13681**	13682	13700	13681.65±3.63
1	**13775**	13793	13811	13780.95±6.03
2	13689	**13887**	13905	13883.90±9.78
3	13693	**13981**	13999	13983.97±7.59
4	14057	14075	**14093**	14092.22±14.0

Figure 4.23: calculated masses for Schiff base formation, Schiff base monohydrate and dihydrate formation. Figures in bold indicate species which have a mass within the error margin of the mass recorded by electrospray mass spectrometry.

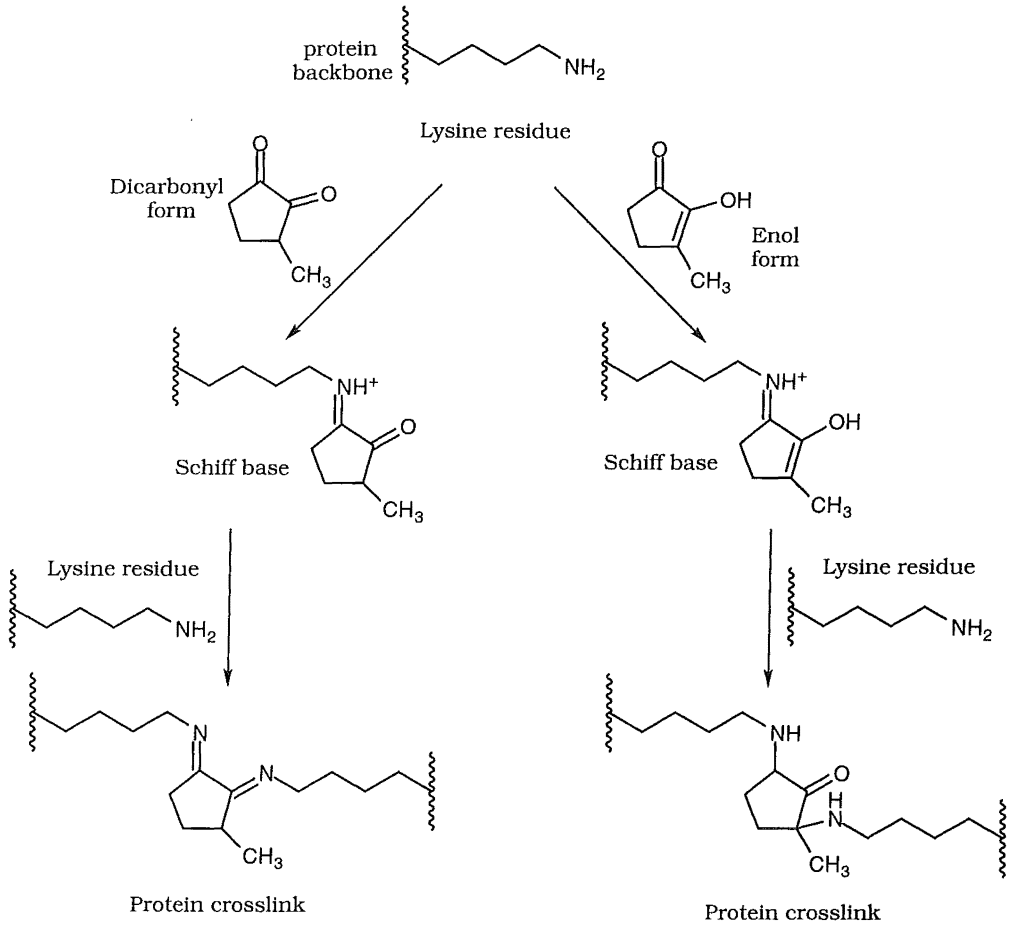


Figure 4.24: two proposed mechanisms for the formation of the Schiff base RNAse A-cyclotene adduct observed by electrospray mass spectrometry.

Whilst other workers have obtained evidence of the Maillard reaction with amino acids and peptides,[4,27] with few exceptions[28,29] direct evidence of Maillard products with proteins remains elusive.

4.9 Summary

The introduction of DHA-mediated protein crosslinks *via* the Maillard reaction, was demonstrated using the model protein, RNAse A. The effect of various reaction conditions, including incubation temperature, pH and protein concentration, on the relative rate of the crosslinking reaction, were examined.

Mechanistic studies provided compelling evidence that the formation of non-disulfide covalent crosslinks between protein monomers was occurring *via* reaction at the lysine residue. Proposed reaction mechanisms are shown in *figure 4.25.* When free amino groups, such as those of lysine, were capped, no crosslinking occurred. The capping of cysteine residues, resulting in the prevention of disulfide bonding, was found to increase the rate of the crosslinking reaction.

Having studied the crosslinking reaction of DHA, the reactions of various breakdown products of DHA were also investigated. The compounds threose, glyoxal and cyclotene were found to crosslink RNAse A to varying degrees. SDS-PAGE analysis of cyclotene-RNAse A reaction products indicated that the relative rate of the reaction was somewhat slower than that of threose, glyoxal and DHA, but that the reaction proceeded very cleanly. Electrospray mass spectrometry of the cyclotene-RNAse A products indicated that the successive addition of four molecules of cyclotene, to the monomeric protein, had occurred.

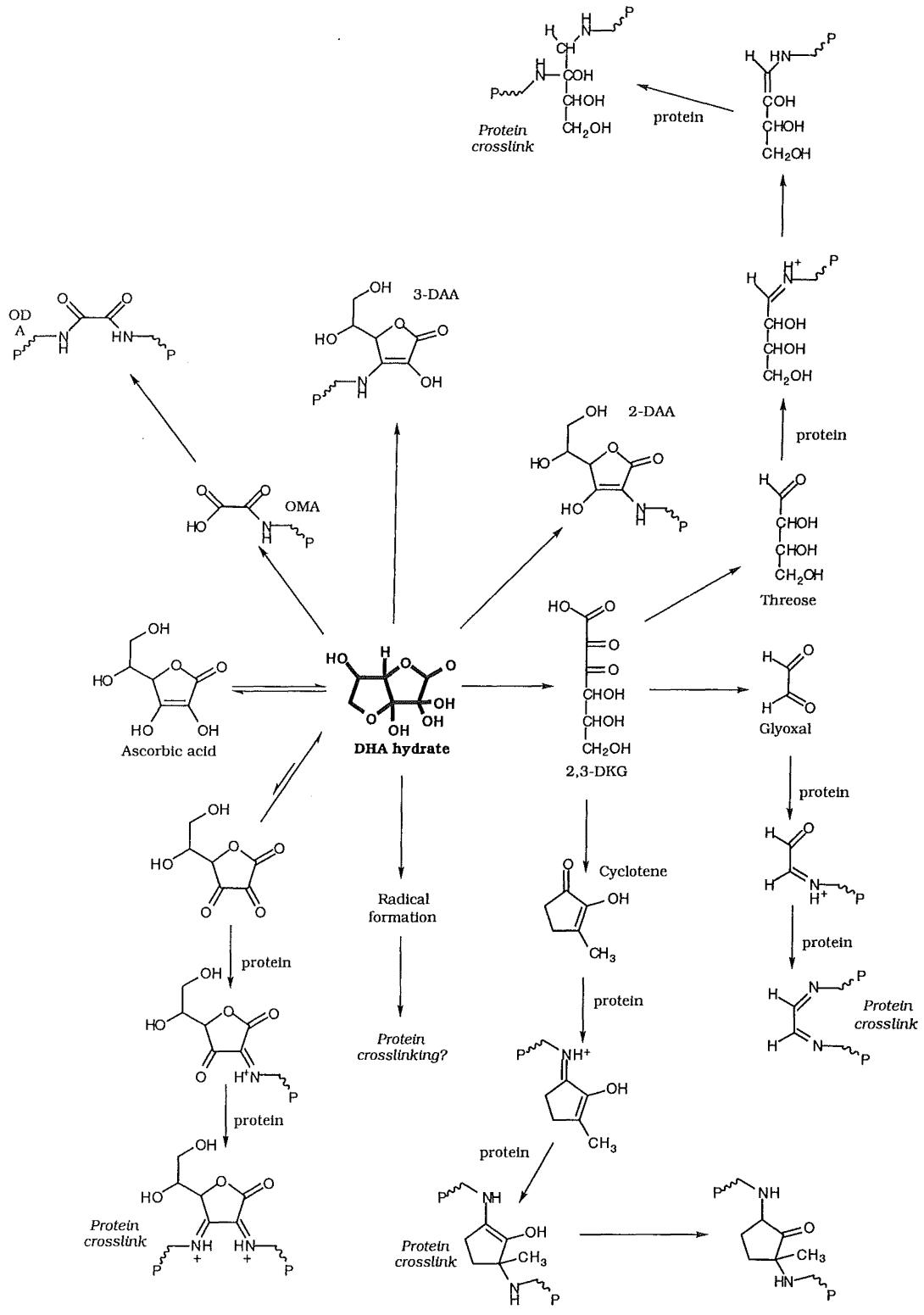


Figure 4.24: possible mechanisms for the formation of protein crosslinks mediated by DHA or its degradation products.

4.10 References

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CROSSLINKING OF WHEAT PROTEINS

5.1 Background

In *chapter four*, the various conditions under which DHA-mediated crosslinks are formed in a model protein, RNAse A, were described. The effect of variables, such as temperature, pH and protein concentration, on the rate of the reaction were also discussed. The next stage of this research was to explore the relationship between the crosslinking reactions of the model systems and those of food proteins. We were particularly interested in the effects of the crosslinking reaction in grain-based foods, for example in the baking of bread, as will be described in *chapter six*.

When flour and water are mixed, some of the protein components combine to form gluten. Gluten proteins are made up of polymers of discrete protein subunits which are held together by a wide range of chemical interactions including hydrogen bonds, Van der Waals interactions, hydrophobic associations and disulfide bonds.[1]

With continued mixing, the gluten proteins are forced to interact, developing an extended network of gluten proteins, which provides the structural framework of the dough.[2] The strength and elasticity of this framework determines the amount of gas retained during both proofing and baking, leading to the various properties of the resulting baked loaf, such as its volume and texture.[3]

Excessive mixing, however, appears to destroy the continuous network of gluten proteins of the developed dough, and leads to a decrease in the quality of the baked loaf. The quality of the final product, therefore, requires the creation of an optimum dough structure through the interaction of dough proteins with other flour components.[4]

The gluten proteins can be divided into two classes of proteins, the gliadins and the glutenins. Gliadins are monomeric proteins of relatively low molecular weight (approximately 40 kDa). Glutenins, on the other hand, are much larger, polymeric proteins which consist of two types of subunits, high molecular weight (HMW) glutenin subunits and low molecular weight (LMW) glutenin subunits. The HMW glutenin subunits are thought to be particularly important in determining the overall breadmaking properties of wheat.[5]

The functional properties of the dough can be improved by the addition of a flour improver. As most flour improvers are oxidising agents, their beneficial effects are generally assumed to result from the oxidation of cysteine residues in the protein, as discussed in *chapter two*. The resulting disulfide crosslinks are thought to further strengthen the protein network, leading to changes in the dough properties and the quality of the baked loaf.[1,6] Crosslinks between the components of the gluten matrix, particularly involving the HMW glutenin subunits, can have a direct effect on the functional properties of gluten. Therefore, the HMW glutenin subunits were selected as the protein component of this investigation.

Alternative DHA-mediated protein crosslinks, such as those introduced to the model protein RNAse A, may also occur in grain-based foods and could further contribute to the strengthening of the protein network. The occurrence of such crosslinks, between HMW glutenin subunits, and their effect on the properties of the protein, and the resulting baked products, have not been thoroughly studied.

Therefore, the first half of this chapter will investigate the importance of the protein crosslinking reactions described in *chapter four*, in grain-based foods, particularly as regards the HMW glutenin subunits.

We have proposed that the DHA-mediated, non-disulfide protein crosslinks described in *chapter four* may have a profound effect on the functionality of the protein and lead to an improvement in the dough and baked properties of grain-based foods. If this is the case, then alternative methods for the introduction of non-disufide crosslinks into food proteins, *via* a non-oxidative mechanism, may also improve the quality of grain-based foods. In order to investigate this possibility, the latter half of this chapter will investigate an enzyme-mediated method for the introduction of non-disulfide crosslinks, into the HMW glutenin subunits, which are of a similar nature to those proposed to occur in the presence of DHA. This work will then be expanded, in *chapter six*, to include the effects of such enzyme-mediated crosslinks, during bread manufacture, on both the dough properties and those of the baked loaf.

5.2 *Investigation into the Maillard reactivity of dehydroascorbic acid with high molecular weight glutenin subunits*

Wheat proteins can be fractionated by a variety of methods, including gel filtration,[7] ultracentrifugation,[8] RP-HPLC,[9] SE-HPLC[10] and capillary electrophoresis.[11] The most commonly used techniques, due to their simplicity and low cost, are procedures based on differences in solubility between the polymeric glutenin proteins and the monomeric proteins, such as the gliadin and the albumin proteins, in various solvents and at different pH levels. These techniques include the Osborne solubility fractionation,[12] pH precipitation,[13] and various other solvent fractionation procedures.[14,15]

The extraction technique, selected for use in this study, was that of Sutton,[16] which relies on the solubility differences of the reduced

wheat proteins in 60% propanol. This procedure allowed the extraction of HMW glutenin subunits from flour, with relative ease and with few contaminations.

For the initial investigation into the Maillard reactivity of DHA with HMW glutenin subunits, we used similar methodology to that of the RNAse A-DHA model systems described in *chapter four.* Unlike the highly water-soluble RNAse A, HMW glutenin subunits are effectively insoluble in water, partly due to their considerable size. As such, the HMW glutenin subunits are particularly difficult to work with. Various efforts have been made to increase the solubility of these proteins, most of which employ organic solvents containing varying amounts of salt.[17,18] Although these techniques are valuable for the extraction of wheat proteins, they do not provide a useful solvent for the modelling of reaction conditions such as those occurring during food processing. Therefore, modifications of the previous methodology were required.

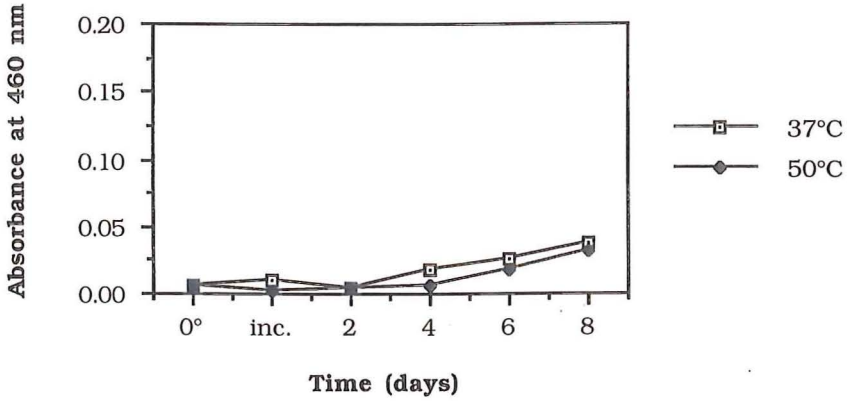
Whereas the majority of the RNAse A reaction systems were incubated in distilled water or simple buffers, the glutenin subunits required a buffered solution (pH 6.8) containing both the denaturant, urea, and the reducing agent, DTT, to aid dissolution. Three concentrations of HMW glutenin subunits were incubated with DHA at either 37°C or 50°C. In each case, a control tube containing a buffered solution of HMW glutenin subunits was also run.

Visual inspection of the products of the reactions showed a gradual increase in the presence of coloured products. *Figure 5.1* shows an increase in browning from day two to day eight. The relative intensities of colour formation were less than that of the associated RNAse A-DHA systems (*figure 4.3*), implying that the rate of the browning reaction, involving the HMW glutenin subunits, was somewhat slower. The control tube, on the left of the figure, remained colourless throughout the reaction, demonstrating that DHA must be present for browning to occur.

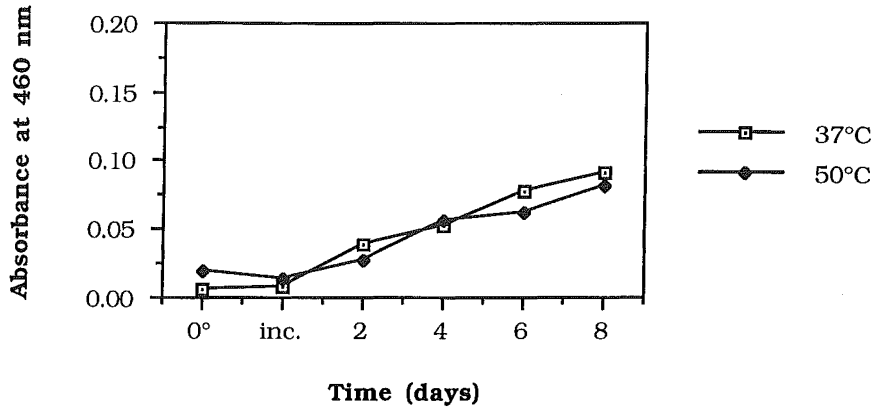
Figure 5.1: increased formation of coloured products with increasing time.

The Maillard reactivity of the DHA-HMW glutenin subunits reaction was further examined by monitoring the UV absorbance at 460 nm.

a) reactions of DHA with HMW glutenin subunits at a protein concentration of 10 mg/mL



b) reactions of DHA with HMW glutenin subunits at a protein concentration of 25 mg/mL



c) reactions of DHA with HMW glutenin subunits at a protein concentration of 50 mg/mL

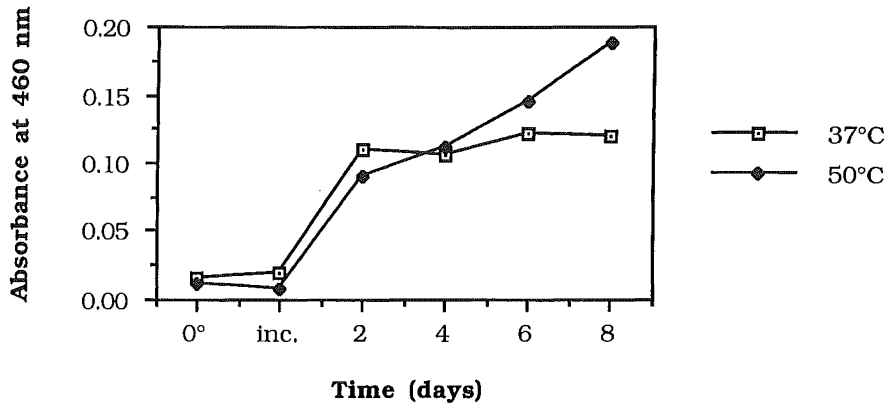


Figure 5.2: comparison of the Maillard reactivity of DHA with HMW glutenin subunits when incubated at either 37°C or 50°C, at a variety of concentrations.

In *figure 5.2* it can be seen that the absorbance at 460 nm increases with both increasing protein concentration and incubation temperature. Whereas peak absorbance in the reaction between RNAse A and DHA occurred at day two, the reaction between DHA and HMW glutenin subunits continues to increase over time without the formation of insoluble protein aggregates. This confirms that the

browning reaction of HMW glutenin subunits occurs, but at a slower rate than that of the RNAse A reaction systems.

Having shown an increase in absorbance at 460 nm, demonstrating the occurrence of Maillard chemistry, the formation of non-disulfide crosslinks was again investigated using SDS-PAGE under reducing conditions.

As HMW glutenin subunits are considerably larger in size than RNAse A, it was necessary to modify the methodology developed for the analysis of non-disulfide crosslinks in the DHA-RNAse A reaction systems. The porosity of the SDS-PAGE gel was manipulated such that the HMW glutenin subunits were able to migrate towards the bottom of the gel. Any crosslinked protein should then appear towards the top of the resolving gel. The acrylamide and bis-acrylamide concentrations were gradually lowered until the optimum concentrations, which allowed this to occur, were found. The optimum load volumes, for the different concentrations of HMW glutenin subunits to be investigated, were also studied, and were found to be the same to those used for the RNAse A-DHA systems, equating to 250 μg of protein.

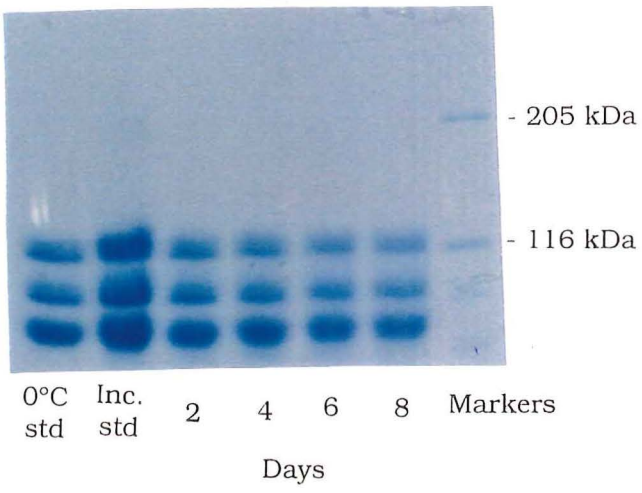


Figure 5.3: SDS-PAGE gel of HMW glutenin subunits, incubated at 37°C for a period of eight days.

Initial studies involved the analysis of samples containing only HMW glutenin subunits which had been incubated at either 37°C or 50°C for eight days. An example of these control gels is shown in *figure 5.3.*

When the HMW glutenin subunits were incubated with DHA under the same conditions, no crosslinked products were observed. Analysis of HMW glutenin subunits incubated with DHA at higher concentrations and temperature, by SDS-PAGE, also did not indicate that crosslinked products had been formed.

To further examine this reaction system, fresh samples from a DHA-HMW glutenin experiment were analysed by SE-HPLC, with diode array detection.

DHA was incubated with HMW glutenin subunits, under standard conditions. Aliquots were removed on the third day and immediately analysed by SE-HPLC, under both reducing and non-reducing conditions. In an attempt to gain some information about the mechanism of the reaction of DHA with HMW glutenin subunits, the above reaction was repeated using HMW glutenin subunits in which the cysteine residues had been capped with 4-VP. In each case, a control was also investigated, identical for each experiment, but with DHA omitted.

Analysis of the products of each experiment indicated that no crosslinking had occurred, possibly reflecting the slow rate of the reaction. A UV spectrum consistent with Schiff base formation was observed at retention time corresponding to elution of the buffer solution. This may indicate that some reaction of DHA, at the amino group of the Tris buffer employed for this series of reactions, had occurred.

Differences in reaction rate, between the RNAse A-DHA reaction systems and those containing HMW glutenin subunits, may be attributed to a number of variables. The most obvious variable which may have affected the rate of the reaction is the difference in lysine

content of the two proteins. Whereas RNAse A contains ten lysine residues, equating to 8 mol% of the protein,[19] HMW glutenin subunits generally contain approximately 0.6-1.2 mol% lysine residues.[20] This difference in amino content, between the two proteins, is likely to have had a pronounced affect on the rate of the Maillard reaction.

Although the reactivity of the HMW glutenin subunits is expected to be lower than that of RNAse A, due to the decrease in available amino groups, the different buffer systems employed for the two reaction systems further complicates any comparison of their reactivities. In order to investigate any differences in reactivity due to the solvent system employed, RNAse A was incubated under the HMW glutenin subunit conditions. Analysis by SDS-PAGE demonstrated that no crosslinking had occurred. However, when RNAse A is incubated in a phosphate/DTT solution, some crosslinking was observed but at a slightly slower rate than that occurring in the absence of DTT. This corroborated an earlier result in which SE-HPLC indicated that DHA had reacted with the Tris buffer. An incubation of DHA in Tris buffer, without protein present, resulted in a UV spectrum consistent with the formation of a Schiff base. This further confirmed our conclusion that DHA was preferentially reacting with the amino group of Tris. However, this does not fully explain the UV absorbance, as the absorbance resulting from the reaction of DHA with the Tris buffer was less than that resulting from the reaction of DHA with the HMW glutenin subunits in Tris-HCl. Therefore, there must be some reaction due to the HMW glutenin subunits.

A further difficulty associated with this reaction system arises from the presence of DTT. 1% DTT was added to the reaction mixture in order to aid dissolution of the HMW glutenin subunits. It's presence, however, will have lead to the reduction of a proportion of the DHA present in solution. Although a molar excess of DHA is present in both the 25 mg/mL and 50 mg/mL reaction systems, the 10 mg/mL samples are likely to contain only ascorbic acid, the reduced form of DHA. This reaction system, therefore, should be considered as a control for the crosslinking reaction since it is unlikely that any DHA

was present. It would be expected, however, that the systems containing 25 mg/mL or 50 mg/mL DHA would undergo DHA-mediated crosslinkage, despite the presence of DTT, albeit at a reduced rate. This was confirmed by analysis of the RNAse A-DHA reaction, in phosphate buffer containing 1% DTT, as described above.

The difficulties encountered in this study indicated that the development of new methodology was required for further investigation into the crosslinking reaction of DHA with HMW glutenin subunits. As it was evident from the literature[21,22] that the glutenin subunits are inherently insolubility and that improving their solubility, particularly in the absence of DTT, is not a trivial task, it was decided that further investigation was beyond the scope of this thesis. This work was, therefore, not pursued.

Although the results of this study have not provided conclusive evidence for the formation of protein crosslinks, evidence that Maillard chemistry is occurring has been obtained. The effects of this chemistry on the functional properties of food proteins may be significant in grain-based foods.

Our attention was instead drawn to an alternative, enzyme-mediated mechanism for the introduction of non-disulfide crosslinks to the HMW glutenin subunits.

5.3 *Transglutaminase-mediated introduction of non-disulfide covalent crosslinks to high molecular weight glutenin subunits*

Covalent crosslinks can be formed by either chemical modification or *via* enzyme-catalysed reactions. Previous chapters have focused on the chemical modification of proteins resulting in the introduction of covalent crosslinks *via* the Maillard reaction. As the introduction of covalent crosslinks to wheat proteins is thought to improve the functional properties of the gluten matrix,[1] alternative mechanisms

for their introduction, enzyme-catalysed mechanisms in particular, were explored.

The use of enzymes in food products provides considerable advantages, both technologically and in terms of consumer acceptance. Enzymatic modifications are specific, require milder conditions than those necessary for chemical modification, and are unlikely to lead to the formation of toxic products.

Several enzymes have the potential to improve the quality of grain-based foods. These include protein disulfide isomerase,[23] protein disulfide reductase,[24] polyphenol oxidase[25] and lipoxygenase,[26] all of which catalyse the formation of disulfide crosslinks. Alternatively, lysyl oxidase[27] and TGAse,[24,28-30] which were discussed in *chapter one*, are two enzymes which are known to catalyse non-disulfide crosslinking.

Of these enzymes, only TGAse is known to crosslink proteins in a process entirely independent of the complex redox chemistry that is present in doughs. The functional properties of the crosslinked proteins, have not been studied in detail.

It has been suggested that TGAse-catalysed modifications may also help protect lysine residues, of food proteins, from various chemical reactions, particularly Maillard reactions.[31] TGAse may, therefore, improve both the functional and nutritional properties of food proteins simultaneously. Additionally, the modifications may produce food proteins with better nutritive value by crosslinking different proteins, containing complementary limiting amino acids.[32]

The remainder of this thesis, therefore, is devoted to the study of the nature of crosslinks introduced to HMW glutenin subunits by the enzyme TGAse and their functional effects.

5.3.1 Transglutaminase-mediated crosslinkage

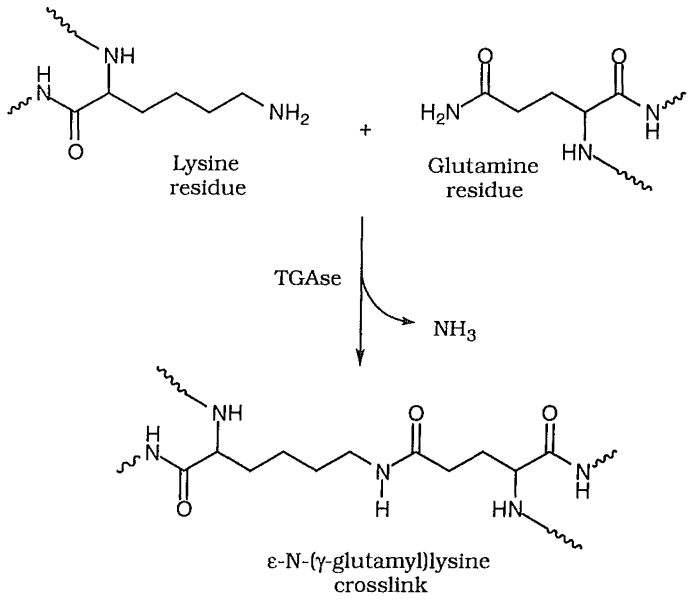


Figure 5.4: the TGAse-mediated formation of ε-N-(γ-glutamyl)lysine *crosslinks.*

TGAse catalyses acyl-transfer reactions between the γ-carboxyamide group of glutamine residues and primary amino groups, such as the ε-amino group of lysine residues, resulting in the formation of ε-N-(γ-glutamyl)lysine crosslinks (*figure 5.4*).[24]

5.3.2 Investigation into the crosslinking reaction of high molecular weight glutenin subunits catalysed by transglutaminase

HMW glutenin subunits were incubated with a high concentration of TGAse, at 37°C, in a buffered solvent (pH 6.8) containing DTT.[33] Aliquots were removed at intervals during the reaction and were analysed by SDS-PAGE. The electrophoretic gel of this reaction showed that immediately after TGAse had been added, all of the glutenin subunits had been crosslinked to such an extent that they were unable to enter the resolving gel. The reaction was, therefore, repeated a number of times, adding progressively less enzyme. At an

enzyme concentration of 95 µg/mL, similar sized material can be seen at the top of the gel after thirty minutes, as shown in *figure 5.5.*

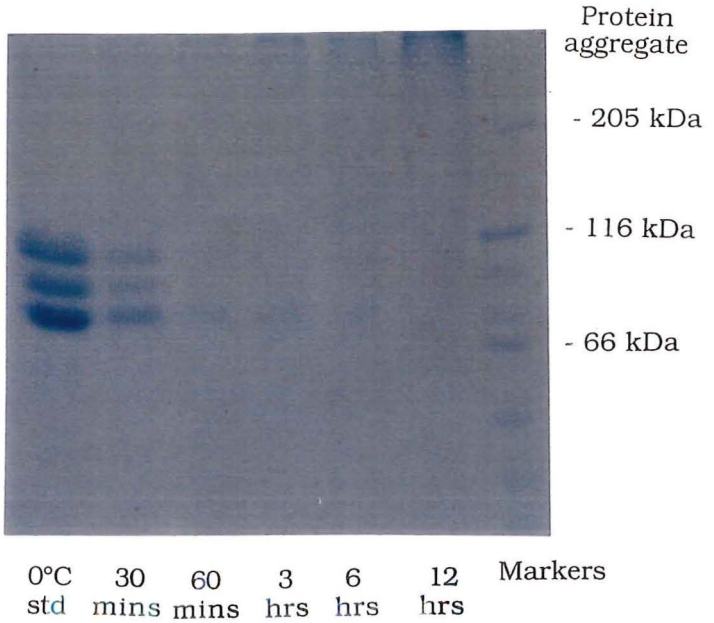


Figure 5.5: SDS-PAGE gel of the products from the TGAse-catalysed crosslinking reaction of HMW glutenin subunits.

Above this concentration, the reaction appeared to have gone to completion within a matter of minutes. However, when the concentration of enzyme, added to the reaction system, dropped below 95 µg/mL, no crosslinking was observed. The reason for this 'all-or-nothing' behaviour is unclear.

Having shown that TGAse is capable of crosslinking the HMW glutenin subunits extracted from flour, we investigated this reaction in dough.

Two doughs were prepared using the same flour as that from which the HMW glutenin subunits, used in the previous experiment, had been extracted. A substantial dose of TGAse was added to one of the doughs while mixing. Both the monomeric and polymeric protein components of each dough were extracted, using 50% propanol.[34] SDS-PAGE analysis of the protein components of each dough is shown in *figure 5.6.*

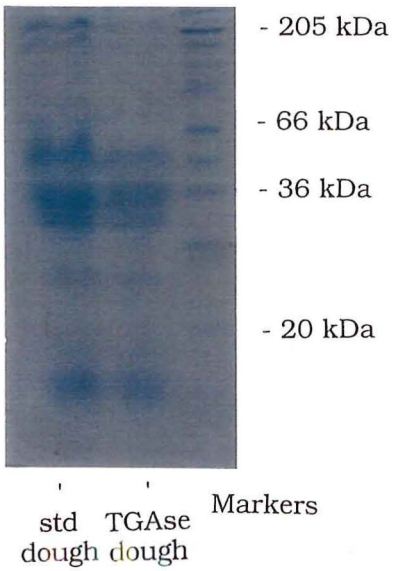


Figure 5.6: SDS-PAGE analysis of the total protein content of a dough containing 5000 p.p.m TGAse compared with that of a standard dough, containing no improver.

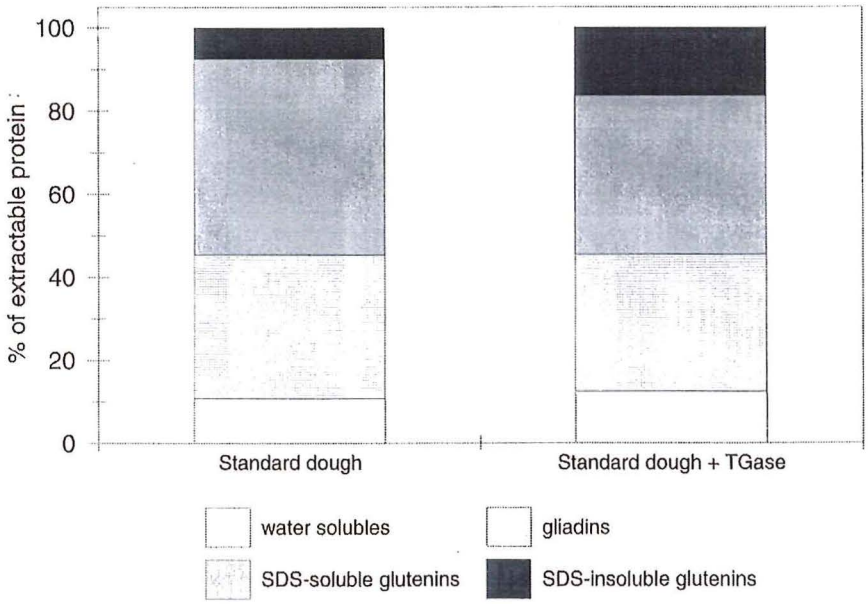


Figure 5.7: SE-HPLC analysis of the protein content of a standard dough compared with that of a dough containing 5000 p.p.m. TGAse.

Analysis of the total proteins shows the loss of the HMW glutenin components of the TGAse-treated dough. No other differences,

between the standard dough and that containing TGAse, were apparent. This is consistent with an increase in mass, due to crosslinking, resulting in an inability to enter the gel. In order to visualise the polymeric material formed during the mixing of the dough, samples were analysed by SE-HPLC, which confirmed the above result. *Figure 5.7* shows an increase in the quantity of polymeric glutenin in the dough to which TGAse had been added, but no change in the gliadin content when compared with a standard dough.

5.3.3 *Assay development for the measurement of transglutaminase activity*

A number of assay techniques can be found in the literature for this enzyme.[35] They include radioactive,[36] fluorometric and electrophoretic assays,[37] as well as colorimetric[38] and immunochemical[39] assays. Most of these assays do not measure crosslinking *per se*, but, instead, monitor another reaction which is catalysed by TGAse, the incorporation of a small amine into a protein (*figure 5.8*).

As we were focussing on the crosslinking reaction, in the first instance we selected an assay which specifically followed crosslinking,[40] rather then amine incorporation, by monitoring the amount of ammonia released during the reaction. We hoped to modify this reaction to give specific information of crosslinking in glutenin proteins.

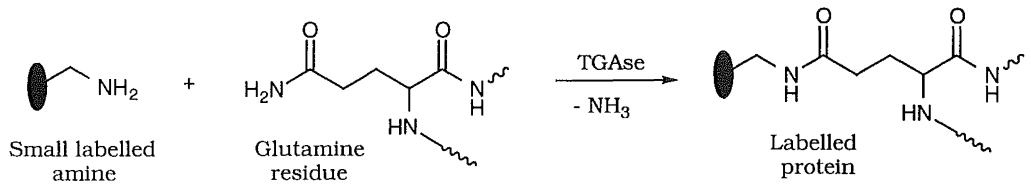


Figure 5.8: the TGAse-catalysed incorporation of a small labelled amine into a protein.

This assay relies on the coupling of the TGAse-catalysed reaction to a second enzyme-catalysed reaction which can be monitored spectrophotometrically (*figure 5.9*). The coupling enzyme is glutamate dehydrogenase, which catalyses the reaction.

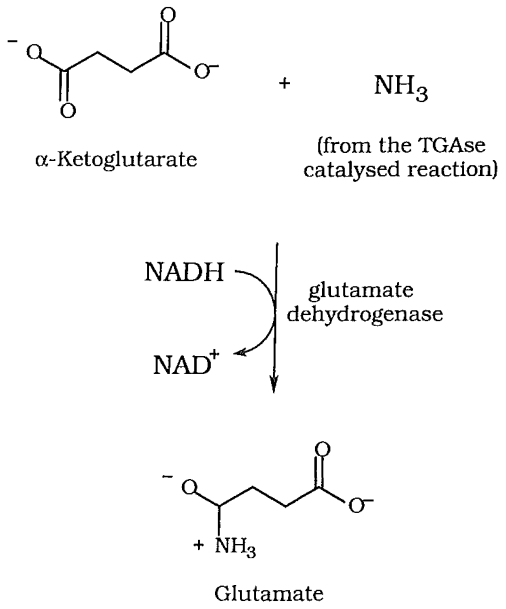


Figure 5.9: TGAse coupled reaction catalysed by the enzyme glutamate dehydrogenase.

The reaction is accompanied by a reduction of the cofactor nicotinamide adenine dinucleotide hydrate (NADH) which can be followed by observing the absorbance change at 340 nm. Reaction conditions must be adjusted to ensure that the rate-limiting step of the reaction is the production of ammonia from the TGAse-mediated crosslinkage of the protein casein. Under the appropriate conditions, the rate of oxidation of NADH is equivalent to the rate of the crosslinking reaction which can, therefore, be monitored.

Established methodology was applied to calibrate the assay,[41,42] in which the rate of the glutamate dehydrogenase reaction is related to the concentration of ammonia. Therefore, if an unknown concentration of ammonia is added to the cuvette, the observed rate can be used to calculate the original ammonia concentration.

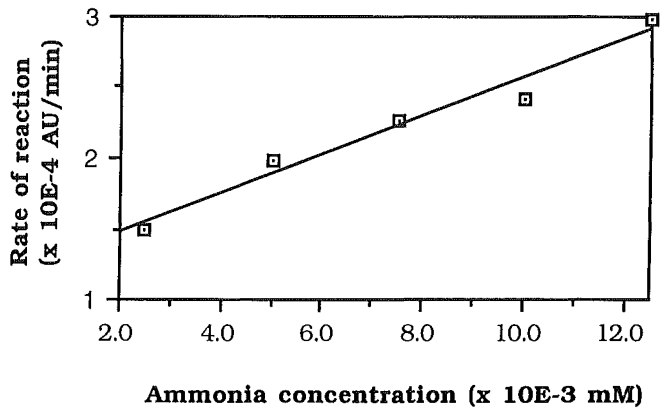


Figure 5.10: standard curve for the calculation of crosslinking activity by the enzyme TGAse. Each point represents the mean value of triplicate readings.

The effect of a range of ammonia concentrations on the absorbance of the assay solution were tested in triplicate. The standard curve obtained from this data is shown in *figure 5.10.*

As the crosslinking reaction, catalysed by TGAse, results in the release of ammonia, the concentration of ammonia released by the reaction can, in theory, be used to calculate the activity of the TGAse. An authentic sample of TGAse, from guinea pig liver, had previously demonstrated activity by this methodology.[43]

Having acquired a standard curve from which the crosslinking activity can be estimated, concentrated stock solutions of microbial TGAse were prepared for testing. The above assay was repeated with the replacement of ammonia solution with a volume of the TGAse solution. A range of volumes were repeatedly tested, however, no activity was apparent. The calculated rates of reaction from these experiments were very small, occurring within the statistical error of the experiment. A number of batches of the enzyme were tested, each returning a similar rate of reaction.

Since the assay proved unreliable, a colorimetric hydroxamate assay was utilised.[44] This assay measures the concentration of hydroxamate formed by the TGAse-catalysed incorporation of hydroxylamine into a peptide containing the amino acid glutamine (*figure 5.11*).

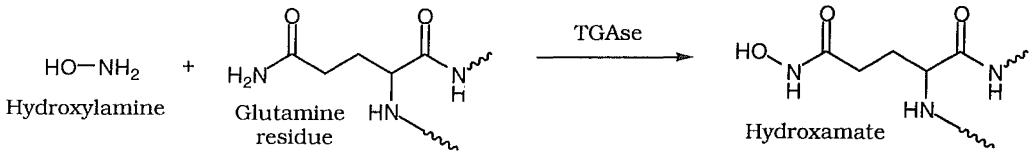


Figure 5.11: hydroxamate formation, catalysed by TGAse.

After reaction, the hydroxamate is chelated by the addition of a ferric chloride reagent, resulting in the observance of a change in absorbance at 525 nm. As ferric chloride cannot chelate hydroxylamine, a change in absorbance is only observed after reaction has occurred (*figure 5.12*).

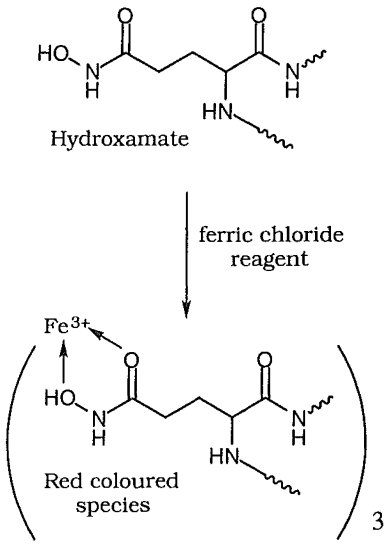


Figure 5.12: the chelation of hydroxamate by ferric chloride.

The above assay was used to test three different batches of TGAse. Results showed that the activity of the enzyme was found to vary between the batches, information which proved valuable in subsequent baking trials.

The affects of TGAse on the properties of dough and baked products were therefore investigated, as described in *chapter six.*

5.4 Summary

Despite the inherent difficulties of working with gluten proteins, HMW glutenin subunits have been shown to react with DHA, in a non-disulfide fashion. The formation of coloured compounds over time, when HMW glutenin subunits are incubated with DHA, provides evidence that Maillard chemistry is occurring. The large size of these proteins combined with their insolubility, makes analysis of the products of this reaction particularly troublesome. Modification of the methodology presented here, especially the use of different buffer systems, may allow the examination of these products.

As crosslinking of HMW glutenin subunits, in the baking of bread, is thought to have a profound influence on the functional properties of the dough, the addition of TGAse, which we have demonstrated to crosslink these proteins in an actual dough, may lead to improvements in both the dough and baked properties of bread.

The enzyme TGAse has been shown to crosslink HMW glutenin subunits *in vitro.* Proteins extracted from bread dough containing TGAse were analysed by SE-HPLC and compared to those from a control dough. TGAse was found to decrease the SDS-soluble glutenin fraction and increase the SDS-insoluble glutenin fraction. The water soluble proteins and gliadins remained unchanged.

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THE EFFECT OF TRANSGLUTAMINASE ON THE FUNCTIONAL PROPERTIES OF WHITE PAN BREAD

6.1 Background

Many studies have been devoted to the detrimental effects of protein crosslinking on the functional properties of proteins during food processing. Much less attention, however, has been paid to the possibility of deliberately introducing such linkages as a method of improving the quality of foods.[1,2]

Bread baking is one of the few areas in which the changes caused by crosslinking have generally been considered desirable.[3] As was discussed in previous chapters, the addition of flour improvers, such as ascorbic acid, is believed to result in the formation of disulfide crosslinks in the gluten matrix. It is these disulfide crosslinks which are thought to be responsible for the resulting improvement in the properties of both the dough and the baked loaf.

This raises the question of whether oxidation itself is exclusively necessary for flour improvement. If the fundamental reason for the change in dough properties is the increase in the level of crosslinking between gluten proteins, then the improvement in dough strength would appear to be only an indirect consequence of oxidation. The direct cause of the improvement in dough properties may simply be a result of an increase in the number of protein crosslinks. If so, alternative mechanisms for the introduction of protein crosslinks, such as that catalysed by TGAse, introduced in *chapter five*, may

provide a way of improving dough strength without the need for oxidation.

The potential value of the enzyme TGAse as a food ingredient in meat and fish products has been recognised for some time, but application has been limited by a lack of an economical source of the enzyme.[2] The search for microbially-derived TGAse, available in bulk from fermentation processes, has only recently come to fruition with the purification and characterisation of a novel TGAse.[4,5] This enzyme differs from all other known TGAses in that it does not require calcium ions for activity. The enzyme is, therefore, ideally suited for food applications.

In the previous chapter, TGAse was shown to catalyse the crosslinking of HMW-glutenin subunits, under both model conditions and in an actual dough. This chapter will continue to examine this reaction, but will be concerned with the affects of TGAse-mediated crosslinking on the properties of the dough and of the baked loaf.

6.2 Use of tranglutaminase in baked products

At the outset of this work, the only source of commercially available TGAse was from guinea pig liver. The scarce source and complicated separation and purification procedures for obtaining this enzyme resulted in it being prohibitively expensive. A further problem with this source of enzyme was that components of guinea pig liver are not particularly appealing food additives! However, we were fortunate to obtain a small sample of microbial TGAse, available *via* the bulk fermentation of micro-organisms, for use in preliminary tests in bread doughs, before it became commercially available.

6.3 Effect of transglutaminase on dough properties

Because TGAse was available in limited quantities only, preliminary work concentrated on small-scale tests and measurements of dough properties. Studying the effect of the enzyme on dough properties

allowed very small scale trials, enabling an estimation of the dose required for the enzyme to have beneficial effects.

Initial observations of the doughs to which TGAse had been added suggested that, when compared with the controls, they were better developed at early stages of resting. An initial dough stickiness was reduced on resting, and the dough developed a rubbery quality that altered the moulding, or shaping, properties of the dough.

In order to quantify the improver response on dough development, a modification of a method due to Frazier[6] was employed. This is a simple and reproducible test that uses a small sample size and is very sensitive to the state of development of the gluten proteins in a dough. The test measures the length of time required by the dough to recover after being compressed by a standard force. That is, it measures the relaxation time of the dough, which indicates the degree of dough development.

Doughs containing 125 g of flour were prepared, with various concentrations of TGAse added. A number of control doughs were also prepared, including doughs with no improver added, as well as doughs containing standard doses of ascorbic acid or ascorbic acid and bromate. Each of the doughs were mixed to a constant work input, ensuring that each dough received the same amount of mechanical mixing energy, so that the development of the various doughs could be compared. The relaxation times of different portions of each dough were then measured at various intervals of the resting stage.

TGAse was found to have a profound influence on the development of the dough, once a certain threshold concentration had been reached. Relaxation times were increased and surpassed those of standard doughs, very early in the resting process, as shown in *figure 6.1*.

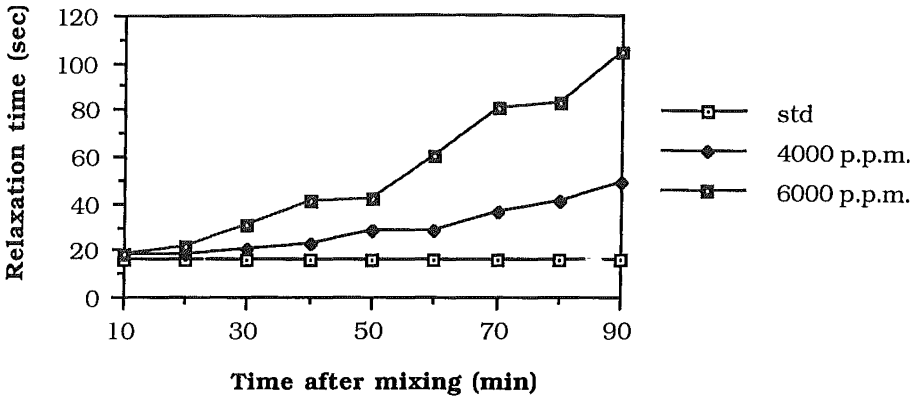

Figure 6.1: the effect of different concentrations of TGAse on the relaxation time of the dough. Each value represents the mean value of triplicate readings.

Ascorbic acid, with or without bromate, also resulted in increased dough relaxation times, although to a much lesser extent than those containing greater than 2500 p.p.m. TGAse. Unlike the treatment with TGAse, the effect due to ascorbic acid did not increase with time. This suggests that the effect of TGAse is cumulative, with more protein crosslinks being formed if the enzyme has had longer to act. Similar effects are, therefore, produced with large doses of enzyme during short rest times, or low doses of enzyme for longer rest times, making TGAse a very flexible processing aid. This is demonstrated in *figure 6.2.*

The fact that relaxation time is increased by the addition of TGAse is strongly suggestive that dough development could be accelerated without such a high requirement for oxidising improvers and high mixing intensity. The change in dough properties that was effected by the enzyme supports the hypothesis that protein crosslinks are important in dough development, and that these can be either disulfide linkages, formed through oxidative processes, or other covalent crosslinks, such as those formed by transglutaminase. Oxidation *per se* is not necessarily essential for dough development.

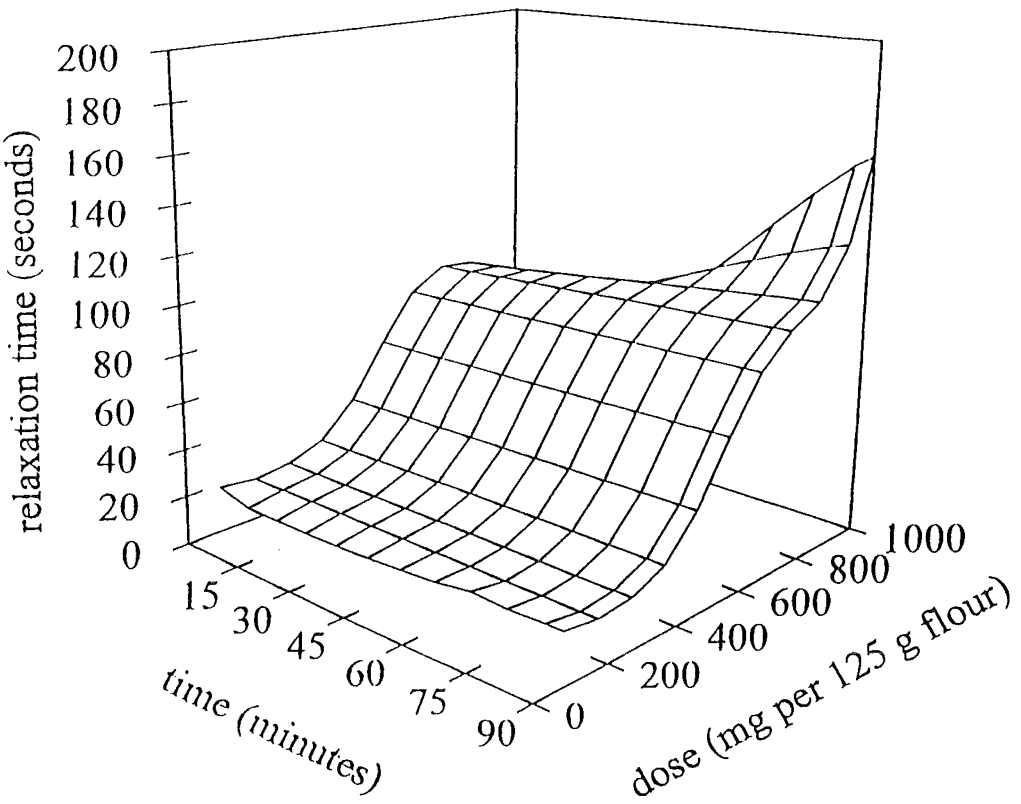

Figure 6.2: a response surface demonstrating the effect of different concentrations of TGAse on the relaxation time over 90 minutes resting.

6.4 *Effect of transglutaminase on water addition*

The ability of a dough to absorb and maintain a larger quantity of water, a 'free' ingredient, is of great interest to bakers. The ability of a dough to absorb a greater volume of water will increase the amount of dough produced and, since this water is mostly retained during the baking process,[7,8] lead to the production of more loaves of bread. The increased yield of bread from the same starting quantity of flour, due to increased water absorption, may correspond to a substantial decrease in the cost in the bulk manufacture of bread, off-setting the cost of the enzyme. Therefore, the water-holding ability of doughs containing various doses of TGAse were investigated.

The volume of water which can be absorbed by a dough is traditionally estimated from the height of the mixing curve which represents the resistance of the dough to mixing. The addition of too much water results in a very 'loose' dough which has reduced resistance to mixing. This is shown by a lowering of the height of the mixing curve.[9] Addition of TGAse to the dough lowered the height of the curve, suggesting that less water should be added. However, immediately after mixing, the TGAse doughs felt too 'tight' and early experiments with TGAse gave loaves that appeared to have suffered from insufficient water addition, even though the water level added was the same as that for the standard doughs. Small trials, adjusting the water level subjectively to give a consistent dough feel, found the doughs containing 5000 p.p.m. TGAse could maintain a water level of 68%, whereas for standard doughs the level remained at 62%. The 6% increase in the water addition to the loaves represents a potential cost saving to the baking industry, which may offset the additional cost of the enzyme.

The reason for the increased water-holding capacity of the dough is not clear, but raises some interesting questions. It is possible that altering the structure of the gluten proteins by crosslinking leads to an increased capacity to hold water, although why this should be true is not obvious. An alternative explanation may be due to a side activity of TGAse: hydrolysis of glutamine residues to glutamate residues in the protein.[10] If this were to occur in the gluten proteins in the dough, the hydrophilicity of the gluten would presumably increase, resulting in a higher affinity for water.

6.5 Effect of transglutaminase on work input requirements

The optimum work input of a dough is the amount of mechanical energy required to mix a dough. It can be defined as the energy required to mix to peak dough consistency[11] or the degree of mixing a flour must receive to obtain peak loaf quality.[12] Lowering the required work input, therefore, also leads directly to cheaper

manufacturing processes. The effect of TGAse on the optimum work input of a dough was evaluated at three concentrations, while maintaining constant water absorption.

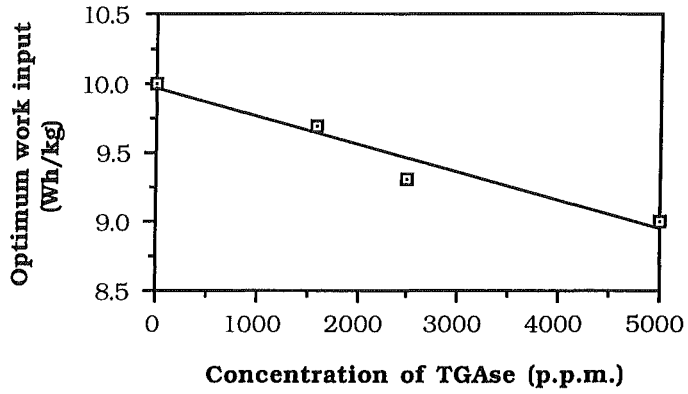

Figure 6.3: the effect of different concentrations of TGAse on the optimum work input of the dough at 150 r.p.m. Each point represents the mean value of triplicate readings.

From *figure 6.3*, it can be seen that the addition of TGAse to the dough lowers the optimum work input. This is consistent with the view that one of the beneficial results of the intensive mixing required for modern breadmaking practices, is the formation of disulfide crosslinks. The formation of crosslinks by another mechanism, therefore, lowers the work input requirement.

Subsequent work[8] showed the effect was more pronounced at 75 r.p.m. That TGAse had a greater effect on the doughs mixed at slower speed presumably reflects the fact that the mixing time was longer, and the enzyme had more time to act. However, the baking quality of the doughs mixed at 75 r.p.m. was severely decreased, but those mixed at 150 r.p.m. at 5000 p.p.m. TGAse produced loaves comparable to the standards.

That the work input is lower at the lower mixing speed confirms the observations of Frazier.[11] The difference in work input requirements

between a standard dough at 150 r.p.m. and a TGAse treated dough at 75 r.p.m. is substantial, and represents potentially large cost savings.

6.6 *Effect of transglutaminase on the crumb strength of baked loaves*

When doughs containing TGAse were baked, a number of properties were affected. For example, the volume of the loaves was found to increase with increasing TGAse concentration, although, they were generally slightly lower than those containing standard improvers, such as ascorbic acid and bromate. The volume improvement, however, did not occur with all batches of flour. In contrast, the texture of bread containing TGAse received increasing texture scores with increasing TGAse concentration. The addition of 5000 p.p.m. TGAse resulted in a texture equivalent to bread containing standard improvers. The most profound effect of TGAse, however, was on the crumb strength of the bread.

Crumb strength is a measure of the ability of sliced bread to withstand handling. A lack of crumb strength is a common complaint amongst consumers, particularly regarding fresh bread, which is difficult to slice and butter.

Bread, containing one of five different doses of TGAse, were prepared using the 50 gram mechanical dough development (MDD) bake test method. One day after baking, the crumb strength of the central slice of bread was measured as the force required to rupture the bread.[8] The addition of TGAse to the bread dough, at each of the concentrations tested, produced a dramatic improvement in crumb strength when compared with that of the controls (*figure 6.4*). This phenomenon is consistent with TGAse crosslinking the gluten matrix, adding strength to the crumb.

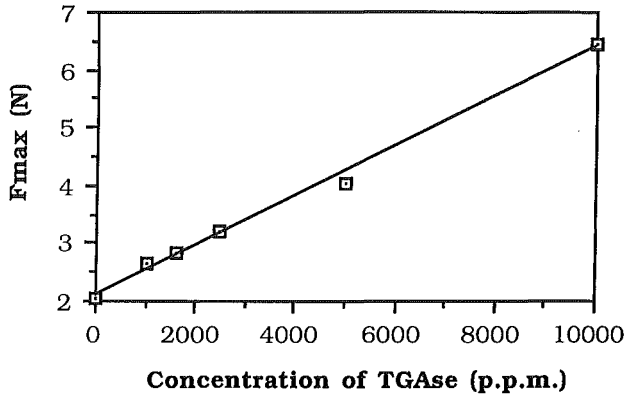

Figure 6.4: the effect of different concentrations of TGAse on the crumb strength of a baked loaf. Each point represents the mean value of triplicate readings.

The experiments reported here are based on current bread manufacturing practices. Since TGAse acts by an entirely different mechanism to oxidising bread improvers, there is scope for altering the whole bread manufacturing process to achieve dough development without investing the same degree of mechanical work.

As larger quantities of TGAse have since become available, the work presented here has led to the investigation of the effects of TGAse on the properties of various other baked products, including croissants, pastries and biscuits. TGAse was again found to have a substantial effect on the properties of these products. This work has led to NZ patent application number 314908, 23rd May 1997.[13,14]

6.7 Summary

TGAse has been shown to have a profound effect on a number of both the properties of the dough and those of the baked loaf. The addition of TGAse leads to an increase in relaxation time, indicating rapid dough development, a decrease in the optimum work input and an increase in the volume of water which can be maintained by the

dough. The resulting baked loaf demonstrates increased crumb strength and improved texture.

These previously unreported effects of TGAse show great promise for its use as a processing aid in the bulk manufacture of white pan bread. Whilst the price is currently prohibitively expensive, it is likely to drop in the near future, as other enzyme companies enter the market.

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SUMMARY

The formation of covalent crosslinks can have a profound effect on the structure and function of a protein. The best characterised form of crosslinking is that of disulfide bonding. However, there are also a number of well-characterised non-disulfide crosslinks, such as those occurring in collagen and elastin.

Covalent crosslinks can be introduced to a protein either enzymatically or *via* various chemical reactions. An example of the latter is the introduction of protein crosslinks as a result of the Maillard reaction. This reaction involves the reaction of free amino groups, such as the ε-amino group of lysine, with carbonyl-containing compounds, such as DHA. The chemical mechanism by which these crosslinks occur is far from understood. It was the primary aim of this thesis, therefore, to investigate this crosslinking reaction and to examine the effect of various reaction conditions on the rate of crosslink formation. The chemistry undertaken in this research is outlined in *figure 7.1*.

As DHA was an ill-characterised compound, an optimised method for the preparation of DHA was developed. DHA was then characterised by NMR. Crystallisation of monomeric DHA was achieved although, unfortunately the quality of the crystals did not enable structure determination. The stability of the compound was also examined. The structure of cyclotene, a degradation product of DHA, was determined by X-ray crystallography. This proved valuable for later investigations.

Having characterised DHA, its reaction with amino acids was reviewed. The Maillard reactivity of DHA with amino acids was investigated and found to be very much greater than that of ascorbic acid or xylose. A method was developed for the separation of the products resulting from the reaction of DHA with amino acids. This method led to the identification of two reaction products: ascorbic acid and 2,2'-nitrilodi-2(2')-deoxyascorbic acid.

DHA was then reacted with the model protein, RNAse A, and was shown to mediate the formation of non-disulfide covalent crosslinks. The rate of the crosslinking reaction was found to increase with increasing time as well as increasing protein concentration and incubation temperature. pH was also found to effect the rate of the reaction, with a greater degree of crosslinking occurring in the more acidic solvent. This was most likely to be due to the instability of DHA in alkaline solutions. Mechanistic studies supported our hypothesis that protein crosslinks were formed at the ε-amino group of the lysine residue, as crosslink formation did not occur when the amino groups of RNAse A were unavailable for reaction.

The reaction of RNAse A with various degradation products of DHA was also investigated. Crosslinking was found to occur when RNAse A was incubated with the compounds glyoxal, threose and cyclotene. However, no crosslinking was evident when oxalic acid was incubated with RNAse A. The electrospray mass spectrometric analysis of the products of the cyclotene-RNAse A reaction provided evidence for a Schiff base intermediate in the crosslinking reaction. In this case, successive addition of four molecules of cyclotene to monomeric RNAse A, over time, was demonstrated.

Having examined the crosslinking reactions of DHA with a model protein, the crosslinking reactions of a food protein were investigated. As we were particularly interested in the effect of the reaction in grain-based foods, the HMW glutenin subunits of wheat, were selected for this series of reactions. However, the technical

difficulties encountered in this study did not enable the formation of distinct conclusions.

An enzyme-mediated mechanism for the introduction of protein crosslinks was also investigated. The enzyme selected for this study was microbially-derived TGAse. It was found to catalyse the crosslinking of HMW glutenin subunits at a minimum concentration of 95 μg/mL. Analysis of the TGAse-catalysed crosslinking reaction in a dough indicated that this reaction may also occur during the baking of bread and may effect the properties of both the dough and the baked loaf. Analysis of these effects demonstrated that this reaction does have a profound effect on the functional properties of the gluten matrix, both before and after baking. For example, increased dough relaxation times, the decrease in the optimum work input and an increase in water absorption resulted from the addition of TGAse to doughs.

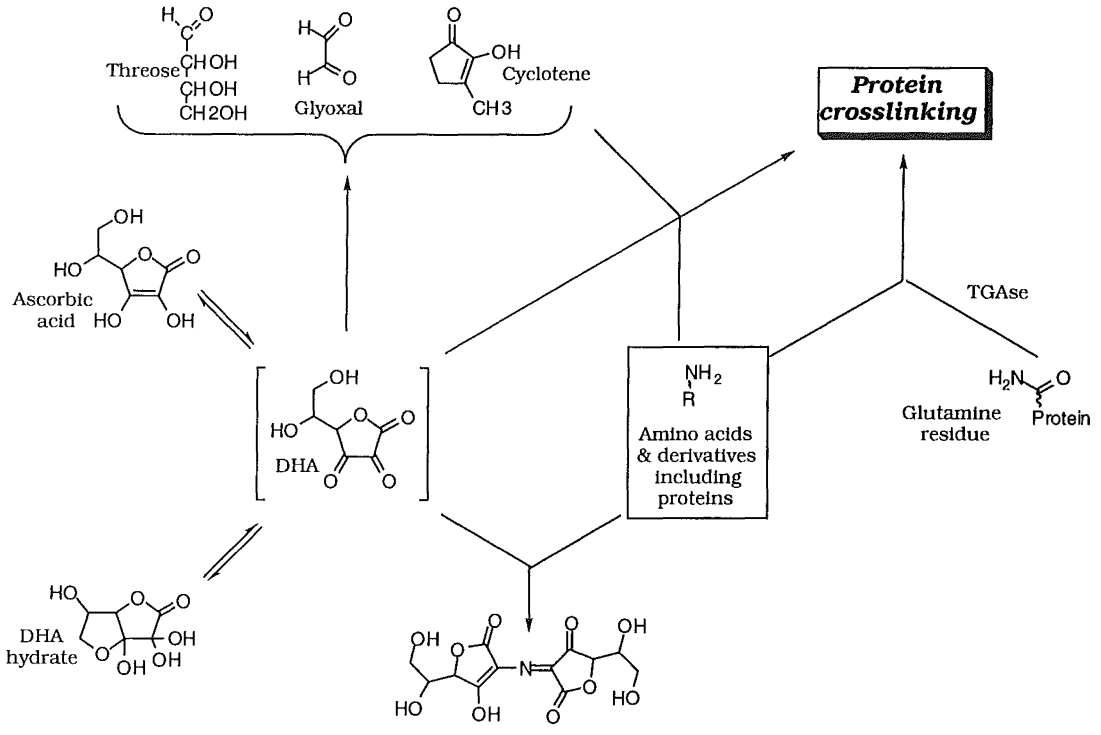


Figure 7.1: outline of the chemistry undertaken in this research.

EXPERIMENTAL

8.1 General methods

Unless otherwise stated, all materials were obtained from Sigma Chemical Company Ltd., Aldrich Chemicals or BDH Laboratory Supplies and were generally of analytical grade. Solvents were purchased from BDH and were of analytical grade.

Ribonuclease A (RNAse A) was Type XII-A (from bovine pancreas) and was obtained from Sigma Chemical Company Ltd.

Methanol was dried by distilling over magnesium methoxide immediately prior to use.

Water for high performance liquid chromatography (HPLC) was produced with a Milli-Q Water Purification System (Millipore).

Thin layer chromatography (TLC) was performed on Merck DC-Plastikfolien Kiessegel 60 F_{254} 20 cm x 20 cm plastic-backed plates. Visualisation was afforded by either ultra-violet light, ninhydrin spray or 2,4-DNP spray.

High speed centrifugation was performed on an Eppendorf Centrifuge 5403, on a small scale (<1.5 mL) at up to 15000 r.p.m., and on a large scale (<50 mL) at up to 5000 r.p.m.

Low speed centrifugation was achieved using a benchtop microcentrifuge supplied by Qualitron Inc.

The whirlimixer was supplied by Fisons Scientific Apparatus Ltd.

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Unity 300 instrument at 23°C operating at 300 MHz for ^{1}H nuclei and at 75 MHz for ^{13}C nuclei, or on a Varian XL 300 instrument. Peaks are quoted in p.p.m. Multiplicities are denoted as singlet (s), doublet (d), triplet (t), quartet (q), doublet of doublets (dd) or multiplet (m).

Ultraviolet (UV) spectroscopy was carried out on a Hewlett Packard 2452A Diode Array Spectrophotometer interfaced with a personal computer running HP 89532A UV-visible operating software.

Infrared spectroscopy (IR) was performed on a Perkin Elmer 1600 FTIR Spectrophotometer or on a Shimadzu FTIR-8201 PC Spectrophotometer. Maxima were recorded in wave numbers (cm^{-1}) and denoted weak (w), medium (m) or strong (s).

Unless otherwise stated, all pH measurements were performed using Panpeha multicolour pH strips - special and universal indicator paper (pH 0-14). Alternatively, pH measurements were performed using a Jenway 3020 pH meter fitted with a Schott Gerate N37BNC electrode, calibrated against standard buffers at pH 4,7 and 9.

Melting points (M.Pt.) less than 200°C were recorded on a Reichert Hotstage microscope and are uncorrected. Melting points greater then 200°C were measured using an Electrothermal melting point apparatus and are uncorrected.

Polyacrylamide gel electrophoresis (PAGE) were routinely run using a Bio-Rad 300 Power Pack. Gel boxes and glass plates for use in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were provided by technical services of Canterbury University. Urea polyacrylamide gel electrophoresis (urea-PAGE) was routinely run using a Gradipore Micrograd electrophoresis unit which was fitted with a circulation pump and a cooling coil. Glass plates were again provided by technical services.

Electrospray mass analyses were performed by Wendy Jackson, Technical Officer, Waikato University. Spectra were recorded in positive ion mode on a VG Platform II mass spectrometer employing a quadrupole mass filter with a *m/z* range of 0-3000. Injection was *via* a Rheodyne injector fitted with a 10 μL sample loop. A Thermo Separation Products SpectraSystem P1000 LC pump delivered the solution to the mass spectrometer source (60°C), and nitrogen was employed both as a drying and nebulising gas. Sample components were identified by manual peak picking using the Transform data processing method included with Masslynx 2.0 software. Electrospray mass spectrometry data are included in *appendix two.*

Heating blocks were made and supplied by the mechanical workshop of the Canterbury University Chemistry Department.

Transglutaminase (TGAse) was obtained from Amcor Trading Pty. Ltd, Melbourne, Australia.

Crude cyclotene hydrate was a kind gift from Dr D.K. Weerasinghe, Group Leader, Organic Chemistry, Applied Research, Firmenich Incorporated, 250 Plainsboro Road, Plainsboro, NJ 08536.

High molecular weight glutenin subunits, containing protected cysteine residues, were a kind gift from Dr A.C. Bekkers, TNO Nutrition and Food Research, The Netherlands.

High performance liquid chromatographic (HPLC) analyses were achieved using one of three systems.

1 The first system consisted of a Waters 600 solvent delivery/control system with a Waters WISP712 automatic sample injector and a Waters 484 UV-visible detector. The column temperature was maintained with a Waters column heater and chromatographic traces were recorded using the Waters Maxima 820 software operating on a personal computer.
2 A second Waters system consisted of a Waters 626 solvent delivery/control system with a Waters WISP717-plus automatic

sample injector or a Waters 2690 "Alliance" solvent delivery/control/automatic sample injector system and a Waters 996 diode array detector. Instruments were controlled and data were recorded using the Waters Millennium 2010 software (version 2.15) operating on a personal computer.

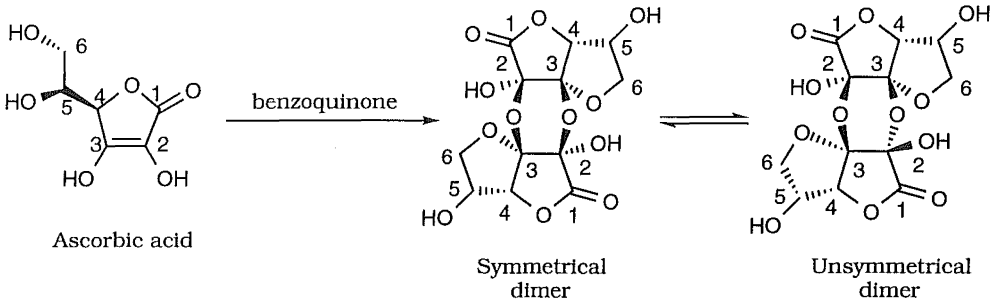
3 Alternatively, analyses were carried out using a Philips PU4100 Liquid Chromatograph System equipped with a Philips PU4120 Diode Array Detector interfaced to a personal computer running Philips PU6003 Diode Array Detector System Software (V3.0) and a colour plotter.

Commercial bread baking flour was purchased from Champion flour mill and stored at -20°C. Its protein and ash contents were 11.4% and 0.54% (on a 14% moisture basis) respectively.[1,2]

Bread ingredient were as follows: Saxa plain salt, Chelsea sugar and Pinnacle yeast from NZ Food Industries Ltd, PO Box 2945, Christchurch, New Zealand.

8.2 *Experimental for work described in chapter two - Preparative chemistry*

8.2.1 *Preparation of dimeric dehydroascorbic acid*[3,4]

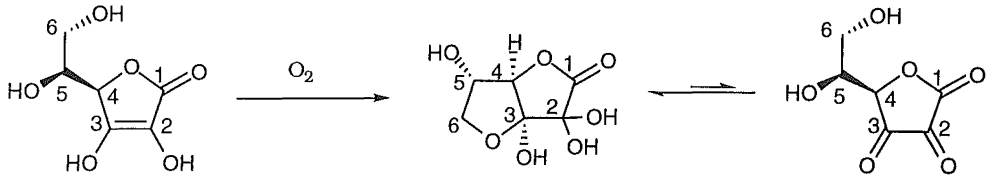


Ascorbic acid (10 g; 56.8 mmol) and dimethyl acetamide (50 mL) were placed in a 200 mL round bottomed flask and warmed gently to dissolve. After cooling to room temperature, a colourless solution of pH~4 was obtained. *p*-Benzoquinone (6.1 g; 56.4 mmol) was added. After shaking vigorously, a very hot, red-brown solution formed which, upon cooling to room temperature, became pale in colour. Crystalline oxalic acid (3 g; 33.3 mmol), dissolved in formic acid (100 mL), was added to the solution. The flask was stoppered and shaken vigorously. Bis-dehydroascorbic acid spontaneously crystallised. After standing overnight, the fine, white crystals were filtered, washed with acetone and ether, air dried and placed under vacuum in a desiccator for two days.

Yield 5.05 g, 51%; M.Pt. 198°C (lit. M.Pt.[5] 201-203°C).

^{13}C NMR (DMSO): δ_C symmetrical dimer: 172.97, C_1; 91.46, C_2; 105.58, C_3; 73.16, C_4; 90.00, C_5; 76.56, C_6; assymetrical dimer: 168.49, 169.01, C_1; 99.48, 103.90, C_2; 103.19, 113.59, C_3; 73.16, 73.40, C_4; 88.11, 19.14, C_5; 74.21, 76.56, C_6. (Data consistent with lit. values[6]).

8.2.2 *Preparation of dehydroascorbic acid using oxygen as the oxidising agent*[7]



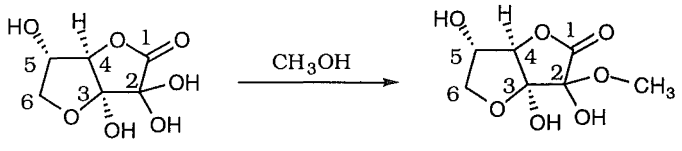
Ascorbic acid DHA hydrate Triketone form of DHA

Ascorbic acid (5 g; 28.4 mmol) was dissolved in the solvent (150 mL), and activated charcoal (5 g) was added. Oxygen was bubbled through the stirred reaction mixture for twenty hours. After removal of the charcoal, *via* filtration through Whatman #2 filter paper, the solvent was removed, under reduced pressure, at a bath temperature of 30°C. The resulting syrup was repeatedly freeze-dried, to remove any residual solvent, before storing at -10°C. DHA was obtained as a colourless syrup.

Solvent:	ethanol:	yield 4.64 g,	94%; R_f 0.65;
	methanol:	yield 3.57 g,	76%; R_f 0.65;
	acetic acid:	yield 3.15 g,	67%; R_f 0.65;

^{1}H NMR (D_2O): δ_H 4.06, dd, 1H, $J_{6a,6b}$ = 10.2 Hz, $J_{6b,5}$ = 2.4 Hz, H_{6b}; 4.20, dd, 1H, $J_{6b,6a}$ = 10.2 Hz, $J_{6a,5}$ = 5.4 Hz, H_{6a}; 4.48, dd, 1H, $J_{5,6a}$ = 5.4 Hz, $J_{5,6b}$ = 2.4 Hz, H_5; 4.65, s, 1H, H_4. ^{13}C NMR (D_2O): δ_C 175.50, C_1; 93.22, C_2; 107.57, C_3; 74.79, C_4; 89.49, C_5; 78.10, C_6.

8.2.3 *Attempted preparation of the methanol complex of dehydroascorbic acid*[8]



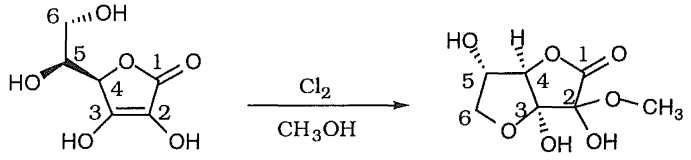
DHA Methanol complex of DHA

Method one

DHA (3.5 g, 20.1 mmol) was dissolved in dry, distilled methanol (20 mL) and refluxed for two hours. The methanol was removed under reduced pressure and the yellow-green syrup (5 mL) was stored at -10°C. As no crystalline material was present after two days, the syrup was further concentrated (2.5 mL). Scratching with a glass rod and cooling to -10°C failed to induce crystallisation.

Spectral data was consistent with the product being a mixture of DHA and ascorbic acid. Authentic samples of both ascorbic acid and DHA corroborated this.

Method two



Ascorbic acid

Methanol complex of DHA

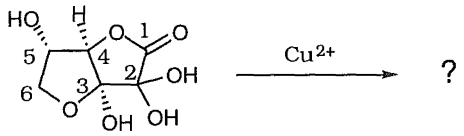
In a round bottomed flask equipped with a stirrer, thermometer, gas delivery tube and gas exit tube, were placed ascorbic acid (1.76 g; 10 mmol), lead carbonate (5.2 g; 20 mmol) and dry methanol (12 mL). The contents of the flask were chilled to -10°C by means of an external cooling bath, and chlorine gas was passed into the stirred suspension at such a rate that the internal temperature remained below -6°C. When the solution became a very pale yellow colour, the addition of gas was stopped. Stirring was continued for a further thirty-five minutes at -6°C. The reaction mixture was then filtered through a thin bed of celite. The filter cake was washed with three 15 mL portions of methanol cooled to -5°C. The clear, colourless solution was treated with a slow stream of hydrogen sulfide producing a powdery white precipitate. Excess hydrogen sulfide was removed by bubbling a brisk stream of nitrogen gas through the suspension. This was again filtered through celite and washed with cold methanol. The solvent was

then removed under reduced pressure, leaving a pale yellow syrup. The syrup was dissolved in methyl isobutyl ketone (30 mL) and stored at -10°C for two days. Colourless, flat crystals were collected by filtration and washed successively with cold methyl isobutyl ketone, then ether, before drying under vacuum. The product was identified as ascorbic acid; none of the desired complex was detected.

Spectral data were identical with those of an authentic sample of ascorbic acid.

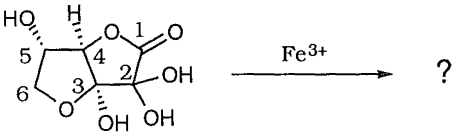
M.Pt.190°C (lit. M.Pt.[9] 190-192°C)

8.2.4 Attempted crystallisation of dehydroascorbic acid salts



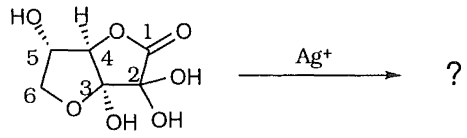
DHA

A mixture of DHA (0.5 g; 2.87 mmol) and either copper (II) chloride dihydrate (0.49 g; 2.87 mmol) or hydrated copper (II) nitrate (0.69 g; 2.86 mmol) was dissolved in ethanol (10 mL). The pH was adjusted to *ca* 5 with sodium hydroxide, and the blue solution was stored at 4°C. No crystalline material was obtained.



DHA

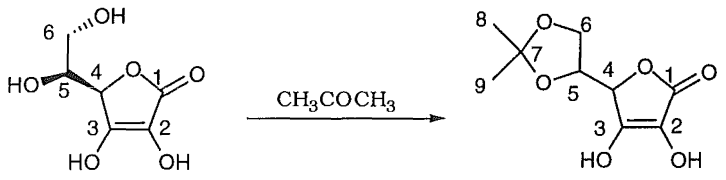
DHA (0.5 g, 2.87 mmol) and iron (III) chloride hexahydrate (0.77 g; 2.87 mmol) were dissolved in ethanol (2 mL). The pH was adjusted with sodium hydroxide such that the hydrated iron salts had just begun to precipitate. The precipitate was redissolved with heating and the solution stored at 4°C. Crystals were not forthcoming.



DHA

DHA (0.5 g, 2.87 mmol) and silver nitrate (0.49 g; 2.87 mmol) were dissolved in ethanol (2 mL) and stored at room temperature. After slow evaporation of the solvent, a silver mirror formed on the inside of the flask indicative of the reduction of silver nitrate to silver.

8.2.5 *Preparation of 5,6-isopropylidene-L-ascorbic acid*[10]



Ascorbic acid

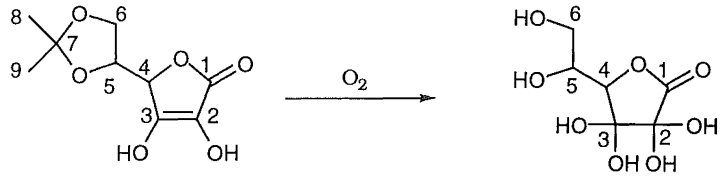
5,6-Isopropylidene
-L-ascorbic acid

Ascorbic acid (10 g; 55 mmol), acetone (40 mL; 550 mmol) and acetyl chloride (1 mL; 15 mmol) were placed in a round bottomed flask with a calcium chloride drying tube and stirred at room temperature for three hours. The flask was stoppered and stored at 4°C for six hours. The solid was collected by filtration and washed with a small portion of cold acetone. The white, crystalline product was recrystallised from acetone/hexane.

Recrystallised yield 10.07 g, 82%; M.Pt. 215°C (lit. M.Pt[11] 217-222°C).

^{1}H NMR (d_6-acetone): δ_H 1.40, s, 3H; 1.41, s, 3H; 4.12, dd, 1H, $J_{6a,6b}$ = 8.5 Hz, $J_{6b,5}$ = 6.8 Hz, H_{6b}; 4.29, dd, 1H, $J_{6b,6a}$ = 8.5 Hz, $J_{6a,5}$ = 6.8 Hz, H_{6a}; 4.47, m, 1H, H_5; 4.82, d, 1H, $J_{4,5}$ = 2.9 Hz, H_4. ^{13}C NMR (D_2O): δ_C 176.10, C_1; 157.77, C_2; 120.01, C_3; 112.91, C_4; 78.31, C_5; 75.03, C_6; 67.18, C_7; 26.91, C_8; 26.09, C_9.

8.2.6 Oxidation of 5,6-isopropylidene-L-ascorbic acid



5,6-Isopropylidene -L-ascorbic acid

DHA

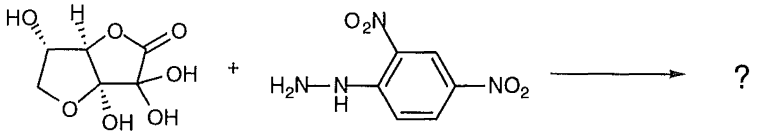
5,6-Isopropylidene-L-ascorbic acid (1 g; 4 mmol) was dissolved in methanol (10 mL). Activated charcoal (1 g) was added and oxygen was passed through the solution for two hours. The charcoal was then removed by filtration and the solution concentrated under reduced pressure.

Yield 0.42 g, 42%;

Samples of the resulting syrup were stored at either -20°C, -10°C, 4°C or 21°C. No crystalline material was obtained.

^{1}H NMR (D_2O) δ_H 4.03, dd, 1H, $J_{6a,6b}$ = 10.2 Hz, $J_{6a,5}$ = 2.4 Hz, H_{6a}; 4.13, dd, 1H, $J_{6b,6a}$ = 9.6 Hz, $J_{6b,5}$ = 5.4 Hz, H_{6b}; 4.42, dd, 1H, $J_{5,6b}$ = 5.4 Hz, $J_{5,6a}$ = 2.4 Hz, H_5; 4.61, s, 1H, H_4. ^{13}C NMR (D_2O): δ_C 173.91, C_1; 91.75, C_2; 106.09, C_3; 88.01, C_4; 73.34, C_5; 76.62, C_6.

8.2.7 2,4-Dinitrophenylhydrazone derivative of dehydroascorbic acid[12]



DHA

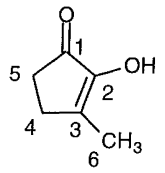
2,4-Dinitrophenyl hydrazine

2,4-Dinitrophenylhydrazine (0.25 g; 1.26 mmol) was suspended in methanol (5 mL). Concentrated sulfuric acid (0.4 mL) was added dropwise. The hot solution was filtered prior to adding DHA (0.2 g; 1.15 mmol) dissolved in methanol (1 mL). The orange solution

became a dark red colour. The precipitate was collected by suction filtration and washed with methanol. Attempted recrystallisation from either ethanol, acetone or glacial acetic acid was unsuccessful. NMR analysis of the resulting red-brown powder suggested that a complex mixture of products had been formed.

M.Pt. 285°C.

8.2.8 Cyclotene hydrate



Enol form of
cyclotene hydrate

Pure crystals of cyclotene hydrate were obtained by recrystallising from water.

^{1}H NMR ($CDCl_3$): δ_H 2.42, br s, 4H, $2H_4$ and $2H_5$; 1.99, s, 3H, $3H_6$; 6.52, br s, 1H, OH. ^{13}C NMR ($CDCl_3$): δ_C 203.22, C_1; 144.85, C_2; 149.09, C_3; 27.13, C_4; 31.90, C_5; 14.27, C_6.

^{1}H NMR (D_2O): δ_H 2.27, m, 2H, H_4 or H_5; 2.35, m, 2H, H_4 or H_5; 1.86, s, 3H, H_6; 3.61, s, 1H, OH. ^{13}C NMR (D_2O): δ_C 208.13, C_1; 148.68, C_2; 153.81, C_3; 28.00, C_4; 32.98, C_5; 14.76, C_6.

X-ray crystallographic data are included in *appendix one.*

8.3 *Experimental for work described in chapter three - Reactions of dehydroascorbic acid with amino acids*

Unless otherwise stated, DHA was prepared using oxygen as the oxidising agent with ethanol as the solvent.

8.3.1 *Comparison of the Maillard reactivity of dehydroascorbic acid with that of xylose and ascorbic acid*[13]

An amino acid (3.3 mmol) was dissolved in 2.5 M phosphate buffer, pH 6.8 (3.3 mL). After the amino acid had dissolved, the solution was transferred to a 10 mL round bottomed flask, packed in ice, which contained either xylose (500 mg, 3.3 mmol), ascorbic acid (581 mg, 3.3 mmol) or DHA (575 mg, 3.3 mmol). The flask was refluxed for one hour. Time zero was taken as the time of mixing. 200 μL aliquots were removed at ten minute intervals and stored on ice prior to UV analysis. A 10 μL sample from each aliquot was made up to 2.5 mL with phosphate buffer and the absorbance read at 460 nm. When the absorbance exceeded 1.5 AU, the solution was diluted and the absorbance calculated accordingly (*table one*).

8.3.2 *Preparation of the dehydroascorbic acid-amino acid model systems*[14-17]

General method

DHA (174 mg; 1 mmol) and an amino acid (1 mmol) were dissolved in either water (10 mL) or methanol (10 mL), and refluxed for forty five minutes. The solution immediately became a deep red colour. A sample was removed for analysis by RP-HPLC using methods two and three (see below). The remaining solution was dried *in vacuo* and stored, in a dry form, at -10°C prior to further analysis by RP-HPLC (*table two*).

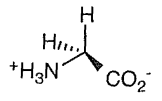
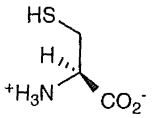
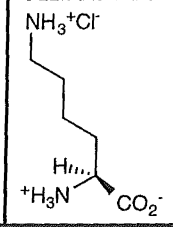
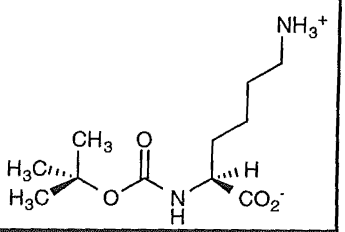
time (mins)	glycine 	cysteine 	lysine-monohydro chloride 	lysine-α-*t*BOC 
DHA - amino acid UV absorbance at 460 nm				
2	0.021	- white ppt	0.159	0.045
10	0.310		0.396	0.276
20	0.495		0.585	0.303
30	0.477		0.626	0.357
40	0.544		0.666	0.520
50	0.558		0.677	0.565
60	0.632		0.683	0.606
ascorbic acid - amino acid UV absorbance at 460 nm				
2	0.002	0.000	0.000	0.183
10	0.006	0.000	0.000	- dark brown oil
20	0.008	0.000	0.002	
30	0.009	0.001	0.002	
40	0.009	0.002	0.005	
50	0.015	0.008	0.009	
60	0.019	0.012	0.013	
xylose - amino acid UV absorbance at 460 nm				
2	0.001	0.001	0.004	1.661
10	0.120	0.001	0.017	- dark brown oil
20	1.575	0.001	0.038	
30	2.483	0.002	0.051	
40	2.702	0.005	0.071	
50	2.781	0.009	0.084	
60	2.826	0.013	0.088	

Table one: comparison of the Maillard reactivity of dehydroascorbic acid with that of xylose and ascorbic acid.

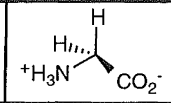
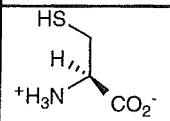
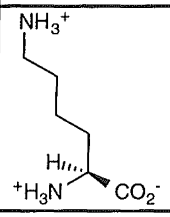
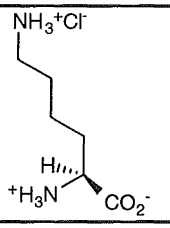
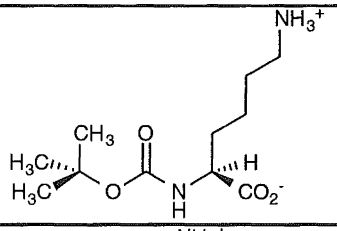
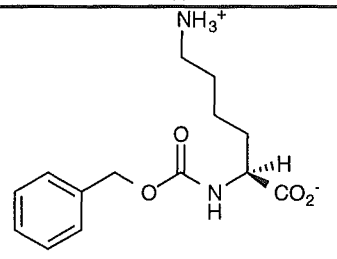
amino acid	structure	amino acid starting weight (mg)	crude yield in methanol (mg)	crude yield in water (mg)
glycine		75	182	317
cysteine		121	110	288
lysine		146	118	202
lysine monohydro chloride		183	205	294
N-α-*t*BOC-lysine		246	352	298
N-α-CBZ-lysine		280	398	344

Table two: preparation of the dehydroascorbic acid-amino acid model systems.

8.3.3 *Analysis of the products of the reactions of dehydroascorbic acid with amino acids by thin layer chromatography*[18,19]

For the separation and identification of products, an *n*-butanol-ethanol-water solvent system (5:3:4) was employed. The samples

(1-2 mg) were redissolved in ethanol (0.5 mL) and spotted onto the lower edge of the thin layer chromatography plate, using a drawn out capillary. The plate was placed in an enclosed tank containing the solvent system (*ca* 10 mL) described above. The plate was removed from the tank when the solvent front was *ca* 0.5 cm from the top of the plate. The red pigment was visually detected (vis.), whereas compounds containing amino groups were visualised by spraying with ninhydrin reagent (NPS). Compounds containing ketone or aldehyde groups, such as DHA, were visualised by spraying with a 2,4-dinitrophenyl hydrazine spray (DNP).

Ninhydrin spray[20]

Ninhydrin (0.25 g) was dissolved in water (100 mL). The samples were separated on a thin layer chromatography plate which was then lightly sprayed with the ninhydrin reagent and placed in an oven at 80°C for two minutes.

2,4-Dinitrophenylhydrazine spray[21]

2,4-Dinitrophenylhydrazine (2 g) was suspended in methanol (100 mL). Concentrated sulfuric acid (4.0 mL) was added and the warm solution was filtered. After the samples had been separated on the thin layer chromatography plate, it was lightly sprayed with the 2,4-DNP reagent.

8.3.4 *Analysis of the products of the reactions of dehydroascorbic acid with amino acids by reversed-phase high performance liquid chromatography*

Method one

HPLC analyses were carried out according to method one as described in *general methods*.

A Brownlee Applied Biosystems Spheri-5 RP18 (220 x 4.6 mm) column fitted with a Brownlee Applied Biosystems guard column (15 x 3.2 mm) was used for these analyses. 20 µL of sample was

injected and spectral data were recorded at four wavelengths (254 nm, 280 nm, 360 nm and 460 nm). The column was maintained at 30°C; a flow rate of 1 mL/min and a data acquisition time of sixty six minutes were used; the solvent gradient was varied from 5% methanol/95% water to 15% methanol/85% water over 60 minutes.

Method two

HPLC analyses were carried out according to method four as described in *general methods.*

The column was an Alltech Analytical Econosphere C_8 (250 x 4.6 mm; 5 µm particle size) fitted with an Alltech C_8 guard column in which, 20 µL of sample was injected. A flow rate of 5 mL/min and data acquisition time of twenty minutes were used. Best results were obtained using isocratic elution with 75% methanol/25% water/0.05% trifluoroacetic acid.

Method three

HPLC analyses were carried out according to method four as described in *general methods.*

20 µL of sample was injected onto a Brownlee Analytical Spheri-5 ODS C_{18} column (250 x 4.6 mm; 5 µm particle size) fitted with a Brownlee C_{18} guard column. A flow rate of between 500 µL/min and 1 mL/min and data acquisition time of up to sixty minutes were used. Best results were obtained using isocratic elution with 75% methanol/25% water/0.05% trifluoroacetic acid.

8.4 *Experimental for work described in chapter four - Protein crosslinking mediated by dehydroascorbic acid*

8.4.1 *General procedure for the incubation of protein with dehydroascorbic acid*

A weighed sample of protein was dissolved in the desired solvent, in an Eppendorf tube. Two aliquots were removed, placed in labelled tubes and stored on ice. These protein-only solutions were control samples; one sample was stored at -10°C, while the second tube was incubated at the reaction temperature, for eight days. The remaining protein solution was transferred to an Eppendorf tube containing DHA. After thorough mixing, the solution was equally divided into four labelled tubes and placed in an incubator at a temperature of either 37°C or 50°C. A tube was removed on alternate days for eight days. The samples were stored at -10°C prior to analysis (*table three*).

	10 mg/mL	25 mg/mL	50 mg/mL
weight of protein (mg)	6	15	15
weight of DHA (mg)	4	10	10
volume of solvent added (μL)	600	600	300
aliqotted volume of solution per tube (μL)	100	100	50
total protein per tube (mg)	1.0	2.5	2.5

Table three: general procedure for the incubation of protein with dehydroascorbic acid.

The reactions of different proteins with DHA were investigated at the three concentrations listed above, under the conditions listed in *table four.*

protein	solvent	temperature (°C)
RNAse A	dH_2O	37
		50
	phthalate buffer (pH 4.0)	37
		50
	phosphate buffer (pH 6.8)	37
		50
	borax buffer (pH 9.1)	37
		50
ovalbumin	dH_2O	37
		50
pepsin A	dH_2O	37
		50
α-amylase	dH_2O	37
		50

Table four: reaction conditions for the DHA-protein model systems.

Preparation of buffer solutions

buffer		mass (g)	pH
0.01 M borax	sodium tetraborate decahydrate	3.800	9.1
0.05 M phosphate	potassium dihydrogen phosphate	3.388	6.8
	disodium hydrogen phosphate	3.534	
0.05 M phthalate	potassium hydrogen phthalate	10.120	4.0

Table five: preparation of buffer solutions.

Standard buffers were prepared by dissolving the above masses of the following compounds in dH_2O (1 L) (*table five*).

8.4.2 *Initial comparison of the Maillard reactivity of dehydroascorbic acid with ribonuclease A under a range of conditions*

Ultra violet absorbance of the supernatant from each reaction sample

UV absorbance (460 nm) of DHA-RNAse A reaction samples at an incubation temperature of 37°C (AU)			
sample	10 mg/mL	25 mg/mL	50 mg/mL
0° standard	0.010	0.009	0.004
incubated standard	0.001	0.011	0.006
day two	0.025	0.237	0.714
day four	0.047	0.253	0.479
day six	0.040	0.323	0.356
day eight	0.029	0.226	0.250

UV absorbance (460 nm) of DHA-RNAse A reaction samples at an incubation temperature of 50°C (AU)			
sample	10 mg/mL	25 mg/mL	50 mg/mL
0° standard	0.003	0.003	0.007
incubated standard	0.005	0.007	0.011
day two	0.053	0.189	0.243
day four	0.013	0.096	0.146
day six	0.007	0.035	0.069
day eight	0.003	0.033	0.055

Table six: UV absorbance of RNAse A samples incubated with DHA under a range of conditions.

Each of the reaction systems carried out in dH_2O were analysed for their Maillard reactivity by monitoring their absorbance at 460

nm. Each of the samples were spun at low speed in a centrifuge and the supernatant of each tube was decanted into a fresh labelled tube. A 30 μL aliquot from each sample was made up to 1.25 mL with dH_2O and its absorbance was read at 460 nm. If the absorbance was greater than 2, the solution was diluted and the absorbance calculated accordingly (*table six*).

Mass of precipitate produced in each reaction sample

mass of precipitate formed in the DHA-RNAse A reaction samples at an incubation temperature of 37°C (mg)			
sample	10 mg/mL	25 mg/mL	50 mg/mL
0° standard	0.8	0.4	1.2
incubated standard	0.3	0.7	0.6
day two	0.3	0.9	0.9
day four	0.4	1.7	1.2
day six	0.4	1.6	2.4
day eight	0.4	1.9	2.5
total protein per tube (mg)	1.0	2.5	2.5

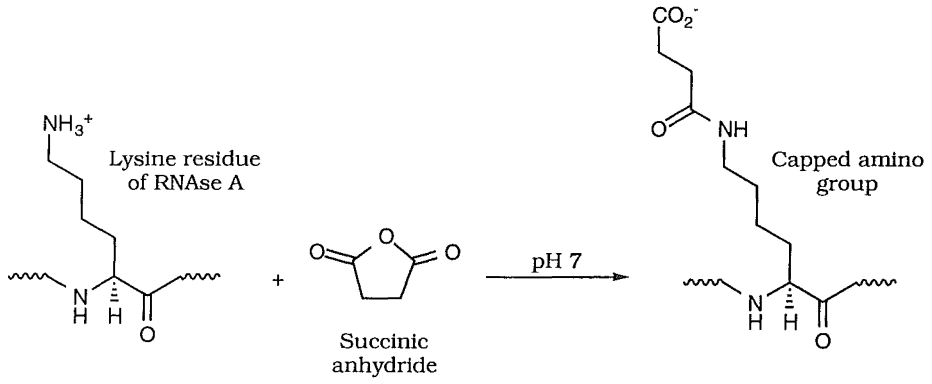
mass of precipitate formed in the DHA-RNAse A reaction samples at an incubation temperature of 50°C (mg)			
sample	10 mg/mL	25 mg/mL	50 mg/mL
0° standard	0.4	0.3	0.4
incubated standard	0.1	0.5	1.4
day two	0.7	2.1	0.5
day four	0.8	2.0	2.1
day six	0.7	2.4	2.6
day eight	0.9	2.4	2.2
total protein per tube (mg)	1.0	2.5	2.5

Table seven: mass of precipitate produced in the DHA-RNAse A model system under a range of conditions.

The supernatant of each of the samples was removed as described above. The tubes, containing precipitated protein, were then dried under vacuum in a dessicator for twenty four hours. Each of the dried tubes was weighed and its weight recorded. The tubes were washed with concentrated hydrochloric acid and rinsed with distilled water ensuring that no protein remained. The tubes were again dried under vacuum and reweighed. The mass of protein contained in each tube was calculated by subtracting the weight of the clean dried tubes from the initial weight (*table seven*).

8.5 *Urea-polyacrylamide gel electrophoresis method for the analysis of the reaction between dehydroascorbic acid and modified ribonuclease A*[22,23]

8.5.1 *Preparation of partially modified ribonuclease A samples*[22]



Ribonuclease A (5 mg) was dissolved in water to a concentration of 1.5 mg/mL and the pH adjusted to 7.0 with 1.0 M sodium hydroxide. Five portions of succinic anhydride (1 mg) were crushed using a mortar and pestle, before being gradually added to the stirred protein solution, over a period of four hours. Approximately 120 µL of base was added to the protein solution, to maintain a pH of 7 throughout the four hours. When the pH had been stable for more than ten minutes, after the addition of a portion of succinic anhydride, a sample (200 µL) was removed. After four hours, a final portion (5 mg) of succinic anhydride was added. When the pH had stabilised, a sample was removed and stored at -10°C prior to electrophoretic analysis.

8.5.2 *Preparation of samples for analysis by urea-polyacrylamide gel electrophoresis*

A 10 µL aliquot from each of the first four tubes and 15 µL aliquots from the remaining tubes were prepared for electrophoresis by the addition of an equal volume of 2x treatment buffer (No 1). The tubes were vortexed and placed in a boiling water bath for three minutes. They were then gently spun on a low speed centrifuge for twenty seconds before loading onto the electrophoretic gel.

8.5.3 *Preparation of stock solutions for urea-polyacrylamide gel electrophoresis*

Acrylamide monomer solution
(33% acrylamide; 0.28% bis-acrylamide)

acrylamide	132.0 g
bis-acrylamide	0.84 g

dH_2O to 400 mL
stored in a dark bottle at 4°C

Resolving gel buffer
(1.0 M Tris-acetate)

Tris	24.23 g

adjusted to pH 3.5 with acetic acid
dH_2O to 200 mL
stored at 4°C

Tank buffer
(0.05 M Tris-acetate)

Tris	12.11 g

adjusted to pH 3.5 with acetic acid
dH_2O to 2 L

2x Treatment buffer (No 1)

methyl green	25 mg
1 M HCl	1 mL
glycerol	2 mL
dH_2O	2 mL

stored at 0°C

0.1% Coomassie brilliant blue stain

Coomassie brilliant blue	0.1 g
glacial acetic acid	10 mL
methanol	50 mL
dH_2O	40 mL

The above ingredients were stirred together for twenty minutes before filtering through a sintered glass funnel under vacuum

Destain
(5% methanol; 10% acetic acid)

glacial acetic acid	100 mL
methanol	50 mL
dH_2O	850 mL

8.5.4 Preparation of urea-polyacrylamide electrophoretic gels

The homogeneous gel solution was prepared using the recipe below (*table eight*). The crystalline urea was dissolved in the dH_2O with gentle heating. After cooling to room temperature, the acrylamide monomer solution was added and the solution was then degassed under vacuum, using a water aspirator, until bubbles ceased to appear.

Two glass plates were washed with Jif liquid, rinsed with water followed by 70% ethanol, before drying at 80°C for fifteen minutes. Three 1.5 mm Teflon spacers were also washed with Jif liquid, rinsed with 70% ethanol and dried. The plates were taped together, using 3M Scotch brand electrical tape, with the three

Teflon spacers forming the gap into which the gel solution was poured and the plates were then heated at 80°C for a further fifteen minutes. A molten 2% agarose solution was then run down the inside edge of the spacers using a pipetteman

homogeneous gel (11% acrylamide, 0.07% bisacrylamide)	
component	volume/mass
acrylamide monomer	10 mL
1 M buffer stock solution	1.5 mL
dH_2O	9 mL
urea	14.41 g
AMPS	300 µL
TEMED	15 µL
final volume	30 mL

Table eight: recipe for the preparation of urea-PAGE gels.

The TEMED and the freshly prepared AMPS solution were added to the gel solution which was then gently swirled, avoiding the introduction of gas bubbles. This solution was slowly poured into the vertical glass sandwich and the comb was inserted ensuring no air bubbles were trapped beneath it. The glass sandwich was adjusted so that it was perfectly level. The gel was allowed to polymerise for three to four hours.

8.5.5 Urea-polyacrylamide gel electrophoresis of modified ribonuclease A

After polymerisation, the adhesive tape was removed from the bottom edge of the glass plates. The gel assembly was clamped into the gelbox and the reservoir was filled with tank buffer. Any air bubbles trapped under the gel were removed by gentle rocking

of the gel box until the bubbles escaped from the side of the gel sandwich. The comb was gently removed exposing the sample wells. Each of the wells was layered with 15 μL of 2x treatment buffer. The gel box was attached to the power pack and the gel was electrophoresed at a constant voltage of 250 V until the methyl green contained in the treatment buffer had travelled half the length of the gel. The power pack was switched off and each of the boiled protein samples were then carefully loaded into separate wells. The power was again turned on until the dye from the protein samples had reached the lower edge of the gel. After approximately five hours, the power was turned off, the gel apparatus was dismantled and the glass plates were carefully prised apart.

The gel was placed in a tank containing Coomassie brilliant blue stain overnight at room temperature. It was then transferred to a tank containing destain solution. The tank was refilled with fresh destain solution regularly, until all excess stain had been removed from the gel. It was then possible to photograph the gel and store it in an airtight container.

8.6 Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of the reactions of dehydroascorbic acid with protein

8.6.1 Preparation of samples for analysis by sodium dodecyl sulfate-polyacrylamide gel elctrophoresis

protein concentration (mg/mL)	load volume (μL)
10	25
25	10
50	5

Table nine: load volumes for SDS-PAGE.

An aliquot, of the desired volume (*table nine*), was removed from each of the samples, and placed in a clean, labelled Eppendorf tube. An equal volume of 2x treatment buffer (No 2) was added to each tube and the cap tightly sealed. After being gently vortexed, they were placed in a boiling water bath for three minutes. They were then vortexed again, centrifuged at low speed for twenty seconds and loaded into separate wells in the polyacrylamide gel.

8.6.2 Preparation of stock solutions for sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Acrylamide monomer solution
(29.2% acrylamide; 0.8% bis-acrylamide)

acrylamide	116.8 g
bis-acrylamide	3.2 g
dH_2O to 400 mL	

stored in a dark bottle at 4°C

Resolving gel buffer
(1.5 M Tris-HCl pH 8.8)

Tris	35.3 g
pH adjusted to 8.8 with HCl	
dH_2O to 200 mL	

stored at 4°C

Stacking gel buffer
(0.5 M Tris-HCl pH 6.8)

Tris	3.0 g
pH adjusted to 6.8 with HCl	
dH_2O to 50 mL	

stored at 4°C

Tank buffer

Tris	6.0 g
glycine	28.8 g
10% (w/v) SDS	20 mL

dH_2O to 2 L

2x Treatment buffer (No 2)

1M Tris-HCl pH 6.8	125 µL
10% (w/v) SDS	2.0 mL
glycerol	1.0 mL
2-mercaptoethanol	500 µL
1% (w/v) bromophenol blue	125 µL
dH_2O	750 µL

stored at -10°C

0.1% Coomassie brilliant blue stain

Coomassie brilliant blue	0.1 g
glacial acetic acid	10 mL
methanol	50 mL
dH_2O	40 mL

the solution was stirred for approximately 20-30 minutes, filtered and stored at room temperature

Destain
(5% methanol; 10% acetic acid)

glacial acetic acid	100 mL
methanol	50 mL
dH_2O	850 mL

8.6.3 Preparation of sodium dodecyl sulfate-polyacrylamide electrophoretic gels[24,25]

The gel solution was prepared according to the following recipe (*table ten*). Acrylamide monomer solution was transferred to a clean dry vacuum flask. The resolving gel buffer, followed by the dH_2O, were also pipetted into the flask. The solution was degassed under vacuum, using a water aspirator, until bubbles ceased to appear.

Two glass plates were washed with Jif liquid, rinsed with water followed by 70% ethanol before drying with paper towels. Three 1.5 mm Teflon spacers were also washed with Jif liquid, rinsed with 70% ethanol and dried. The plates were clamped together, using two bulldog clips along each edge, with the three Teflon spacers forming the gap into which the gel solution was poured. A warmed 2% agarose solution was then carefully pipetted down the inside edges of the gel sandwich to prevent leakage of the gel solution.

component	stacking gel (3.5% acrylamide, 0.1% bisacrylamide)	resolving gel (12.2% acrylamide, 0.33% bisacrylamide)
acrylamide monomer solution	1.33 mL	10.4 mL
resolving gel buffer	-	6.25 mL
stacking gel buffer	2.5 mL	-
10% SDS solution	1.0 mL	250 µL
dH_2O	6.1 mL	7.9 mL
10% AMPS	50 µL	125 µL
TEMED	25 µL	8.3 µL
Final volume	11 mL	25 mL

Table ten: recipe for the preparation of 12.2% SDS-PAGE gels.

The SDS, TEMED and the freshly prepared AMPS solution were added to the gel solution which was gently swirled so as to avoid the introduction of gas bubbles. It was then slowly poured into the

vertical glass sandwich leaving a space of approximately 4 cm at the top into which the stacking gel was polymerised later. The glass sandwich was adjusted so that it was perfectly level. *n*-Butanol was gently laid over the gel solution by carefully running it down the edge of the side spacers using a pipetteman. The resolving gel was left overnight to polymerise.

Once the resolving gel had set the *n*-butanol was poured off and the top of the gel was rinsed with distilled water. The stacking gel solution was made in an identical fashion to the resolving gel and was poured over the top of the resolving gel. The perspex comb was immediately inserted into the stacking gel mixture ensuring no air bubbles were trapped beneath it. The assembly was left undisturbed for approximately forty five minutes while the gel polymerised.

8.6.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of dehydroascorbic acid-protein reaction systems

After polymerisation, the bulldog clips were removed and the bottom spacer was carefully withdrawn from the glass sandwich. The assembly was clamped into the gel box and both reservoirs were filled with tank buffer. Any air bubbles trapped under the gel were removed by gently rocking the gel box until the bubbles escaped from the side of the gel sandwich. The comb was carefully removed exposing the sample wells. The gel box was placed in the fridge and the leads from the power pack were attached to the electrodes on the gel box. Each of the boiled protein samples were carefully loaded into separate wells. An 8 μL sample of a wide range molecular weight standard (*table eleven*), Sigmamarker, which had previously been mixed with 8 μL of 2x treatment buffer (No 2) and boiled for three minutes, was also loaded into a well. The samples were electrophoresed at a constant current of 30 mA until the bromophenol blue dye, contained in the 2x treatment buffer, had reached the lower edge of the gel. This took approximately four four and a half hours.

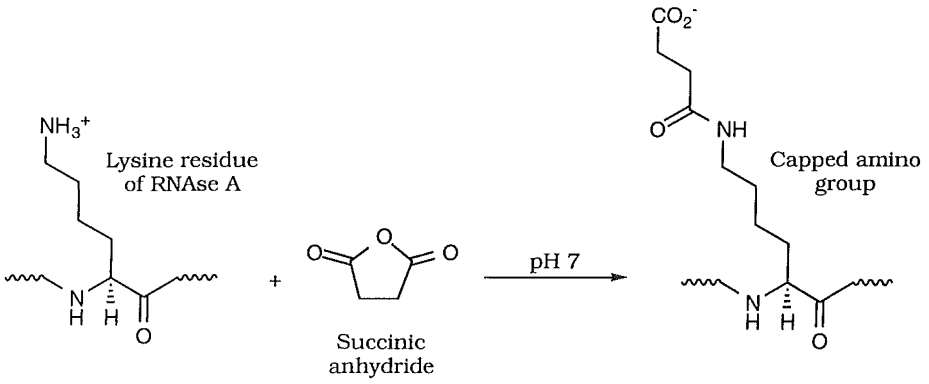
protein	molecular weight (kDa)
myosin, rabbit muscle	205
β-galactosidase, *E. coli*	116
phosphorylase *b*, rabbit muscle	97
fructose-6-phosphate kinase, rabbit muscle	84
albumin, bovine serum	66
glutamic dehydrogenase, bovine liver	55
ovalbumin, chicken egg	45
glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle	36
carbonic anhydrase, bovine erythrocytes	29
trypsinogen, bovine pancreas	24
trypsin inhibitor, soybean	20
α-lactalbumin, bovine milk	14.2
aprotinin, bovine lung	6.5

Table eleven: standard molecular weight protein markers.

The power was turned off, the gel apparatus was dismantled and the glass plates were carefully prised apart. The gel was placed in a tank containing Coomassie brilliant blue stain overnight, at room temperature, before transferring it to a tank containing destain solution. The tank was refilled with fresh destain solution regularly until all of the excess stain had been removed from the gel. It was then possible to photograph the gel before storing it in an airtight container.

8.7 *Investigation into the mechanism of the protein crosslinking reaction*

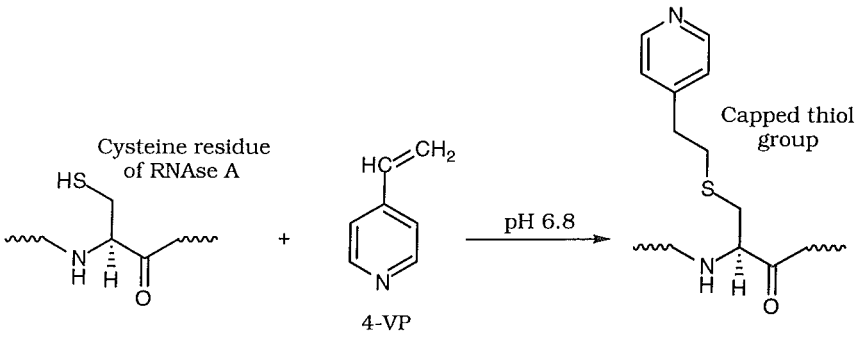
8.7.1 *Reaction of dehydroascorbic acid with ribonuclease A containing capped lysine residues*[22]



Ribonuclease A (20 mg) was dissolved in water to a concentration of 1 mg/mL and the pH adjusted to 7.0 by the addition of 1.0 M sodium hydroxide. Twelve portions of succinic anhydride (2 mg) were added, with constant stirring, over a period of six hours. The pH was maintained at 7 by the dropwise addition of 0.5 M sodium hydroxide. The pH was maintained at 7 between the additions of succinic anhydride, by the addition of *ca* 500 µL of sodium hydroxide was added. The resulting solution was then dialysed overnight at 4°C against stirred distilled water. After dialysis, the protein solution was freeze-dried to a volume of *ca* 1 mL.

The solution was reacted with DHA using the general procedure described previously prior to analysis by SDS-PAGE.

8.7.2 *Reaction of dehydroascorbic acid with ribonuclease A containing capped cysteine residues*[26]



Ribonuclease A (20 mg) was incubated for sixty minutes at 60°C in 0.1 M Tris-HCl buffer (pH 6.8) in 50% (v/v) propan-1-ol (6 mL) which contained 1% (6.48 mM) DTT and 2 M urea. 4-Vinylpyridine (400 μL) was added to the resulting solution which was then incubated at 60°C for fifteen minutes. The protein solution was dialysed overnight at 4°C against dH_2O, with stirring, and then concentrated by freeze-drying.

8.8 *Analysis of dehydroascorbic acid reaction products*

8.8.1 *Preparation of samples for analysis by size exclusion-high performance liquid chromatography*[27]

Two aliquots were removed from each sample and placed in clean, labelled tubes. The first aliquot of each sample was diluted to a volume of 500 μL with 0.05 M sodium hydrogen phosphate containing 0.5 % SDS. To the second aliquot was added an equal volume of a solution containing 4% SDS and 2% DTT. This was then made up to 500 μL as before. The final pH of the samples was 6.9. They were then centrifuged at 10000 x g for ten minutes and filtered through a 5 μm polyvinylidenedifluoride membrane syringe-filter prior to analysis by SE-HPLC (described below).

8.8.2 Conditions for the analysis of dehydroascorbic acid-protein systems by size-exclusion liquid chromatography

HPLC analyses were carried out according to method two as described in *general methods.*

The column used was a TSK-GEL G3000SW column (300 x 7.5 mm) fitted with a TSK-GEL G3000SW guard column (75 x 7.5 mm) which was maintained at 25°C. Injection volume was 20 μL and data acquisition time of up to forty minutes were used. Spectral data were recorded over the wavelength range 190-600 nm.

8.8.3 Preparation of samples for analysis by electrospray mass spectrometry

Samples were diluted 1:10 with 50% (v/v) acetonitrile/water and a volume of 10 μL was injected. A scanning rate of 1 sec/100 Daltons and a skimmer cone voltage of 45 V were used.

8.9 Reaction of ribonuclease A with the degradation products of dehydroascorbic acid

RNAse A was reacted with the compounds cyclotene, threose, oxalic acid and glyoxal at a protein concentration of 25 mg/mL and a reaction temperature of 37°C. Reaction systems were prepared using the general procedure described previously for the reactions of DHA with RNAse A.

The resultant reaction mixtures were analysed by SDS-PAGE, SE-HPLC and electrospray MS, as described previously.

8.10 *Experimental for work described in chapter five - Investigation into the reactions of dehydroascorbic acid with high molecular weight glutenin subunits*

8.10.1 *Extraction of high molecular weight glutenin subunits*[26]

HMW-glutenin subunits were extracted from a commercial bread baking flour purchased from Champion flour mill and stored at -10°C.

Flour samples (100 mg) were extracted with 50% (v/v) propan-1-ol (625 μL), containing 1% (w/v) (64.8 mM) DTT, at 60°C for thirty minutes, with agitation every ten minutes. Following high speed centrifugation (10000 g, 10 mins), an aliquot (500 μL) was removed and placed in a fresh tube. The HMW-glutenin subunits were precipitated by the addition of propan-1-ol (125 μL) containing 1% (w/v) (64.8 mM) DTT. The mixture was vortexed vigorously and allowed to stand at room temperature for thirty minutes. The precipitate was collected by centrifugation and the pellet washed with 60% (v/v) propan-1-ol containing 1% (w/v) (6.48 mM) DTT. The subunits were dried under vacuum and stored at -10°C prior to incubation with DHA.

8.10.2 *Reaction of dehydroascorbic acid with high molecular weight glutenin subunits*

The dried HMW glutenin subunits were dissolved in the required volume of 1 M Tris-HCl buffer (pH 6.8), containing 8 M urea and 1% DTT. The tube was incubated, with agitation, at a temperature of 37°C to aid dissolution. The DHA-protein reaction systems were then prepared according to the general procedure described previously for RNAse A. Three protein concentrations, 10 mg/mL, 25 mg/mL and 50 mg/mL, were each investigated at incubation temperatures of 37°C and 50°C. Control experiments were incubated under the same conditions, with DHA omitted.

8.10.3 Initial investigation of the Maillard reactivity of dehydroascorbic acid with high molecular weight glutenin subunits under a range of conditions

Ultra violet absorbance of each reaction sample

UV absorbance (460 nm) of the samples from the reaction of DHA with HMW glutenin subunits at an incubation temperature of 37°C (mg)			
sample	10 mg/mL	25 mg/mL	50 mg/mL
0° standard	0.006	0.005	0.015
incubated standard	0.009	0.008	0.019
day two	0.007	0.038	0.109
day four	0.018	0.053	0.105
day six	0.025	0.078	0.122
day eight	0.037	0.092	0.119

UV absorbance (460 nm) of the samples from the reaction of DHA with HMW glutenin subunits at an incubation temperature of 50°C (mg)			
sample	10 mg/mL	25 mg/mL	50 mg/mL
0° standard	0.006	0.019	0.012
incubated standard	0.001	0.013	0.007
day two	0.003	0.027	0.090
day four	0.005	0.057	0.112
day six	0.017	0.062	0.146
day eight	0.032	0.082	0.189

Table twelve: UV absorbance of HMW glutenin samples incubated with DHA under a range of conditions.

Each of the reaction systems described above were analysed for their Maillard reactivity by monitoring their absorbance at 460 nm. Samples were prepared as described previously for the reaction of RNAse A with DHA. However, in this case, aliquots from each sample were made up to 1.25 mL with 1 M Tris-HCl (pH 6.8) containing 1% DTT and 8 M urea. Absorbances greater than two

were further diluted and the absorbance calculated accordingly. Absorbances have been corrected by subtraction of the absorption of a non-protein control (*table twelve*).

8.10.4 Preparation of samples for analysis by size exclusion-high performance liquid chromatography[27]

An aliquot was removed from each sample and placed in a clean, labelled tube. Each sample was diluted to a volume of 500 µL with 0.05 M sodium hydrogen phosphate. They were then centrifuged at 10000 x g for ten minutes and filtered through a 5 µm polyvinylidenedifluoride membrane syringe-filter prior to analysis by SE-HPLC (described below).

8.10.5 Conditions for the analysis of dehydroascorbic acid-high molecular weight glutenin reaction systems by size-exclusion liquid chromatography

HPLC analyses were carried out according to method two as described in *general methods.*

The column used was a TSK-GEL G3000SW column (300 x 7.5mm) fitted with a TSK-GEL G3000SW guard column (75 x 7.5mm) which was maintained at 25°C. Injection volume was 20 µL and data acquisition time of up to forty minutes were used. Spectral data were recorded over the wavelength range 190-600 nm.

8.10.6 Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of the crosslinking reaction of dehydroascorbic acid-high molecular weight glutenin subunits reaction systems[24,25]

The preparation of the reaction samples, including samples containing capped cysteine residues, and of the SDS-PAGE stock solutions were as reported for the DHA-RNAse A reaction systems. SDS-PAGE gels were prepared according to the following recipe (*table thirteen*).

component	stacking gel (3.5% acrylamide, 0.1% bisacrylamide)	resolving gel (7% acrylamide, 0.19% bisacrylamide)
acrylamide monomer solution	1.33 mL	6.0 mL
resolving gel buffer	-	6.25 mL
stacking gel buffer	2.5 mL	-
10% SDS solution	1.0 mL	250 µL
dH_2O	6.1 mL	12.3 mL
10% AMPS	50 µL	125 µL
TEMED	25 µL	8.3 µL
Final volume	11 mL	25 mL

Table thirteen: recipe for the preparation of 7% SDS-PAGE gels.

Clean, dry electrophoresis plates were rinsed with 70% ethanol and coated with either the water-repelling agent, Sigmacote, or the bonding agent, methacryloxypropyltrimethoxysilane. After ten minutes, the plates were again washed with 70% ethanol. The gel unit was then rapidly assembled as described previously.

Samples were electrophoresed at a constant current of 30 mA for a set time of seven hours. The gel plates were then carefully prised apart and the gel was stained and destained while attached to the glass plate support.

8.11 Transglutaminase-mediated crosslinking of high molecular weight glutenin subunits

8.11.1 Coupled assay for the measurement of transglutaminase activity

The following stock solutions (*table fourteen*) were freshly prepared each day and stored on ice.

solution	concentration
DTT	1.93 mg/mL
NADH	3.5 mg/mL
glutamate dehydrogenase	0.65 mg/mL
NH_4Cl	0.25 M
casein	50 mg/mL
Tris-acetate (pH 8.0)	0.5 M
α-ketoglutarate	8.4 mg/mL

Table fourteen: coupled assay stock solutions.

The following solution (*table fifteen*) was pipetted into a cuvette and its absorbance was monitored at 340 nm, over ten minutes.

solution	volume (μL)
dH_2O	470
Tris-acetate	200
α-ketoglutarate	120
DTT	40
NADH	40
casein	20
glutamate dehydrogenase	10
dH_2O/NH_4Cl	100
final volume	1000

Table fifteen: coupled assay solution.

The UV spectrophotometer was blanked against dH_2O (1 mL). Analyses of the rate of reaction for each volume of NH_4Cl, were performed in triplicate to obtain a calibration curve for the measurement of TGAse activity (*table sixteen*).

volume of NH_4Cl (µL)	0	10	20	30	40	50
Concentration of NH_4Cl (x 10^{-3} mM)	0	2.5	5.0	7.5	10.0	12.5
calculated rate of reaction (x 10^{-4} AU/min)	0.15	1.42	1.92	2.14	2.37	2.88
	0.20	1.48	1.95	2.27	2.40	3.02
	0.30	1.56	2.11	2.40	2.47	3.07

Table sixteen: effect of different volumes of NH_4Cl on the rate of the coupled assay.

8.11.2 Hydroxamate assay for the measurement of transglutaminase activity[28,29]

solution	concentration
CBZ-L-glutaminylglycine	100 mM
Tris-acetate (pH 6.0)	200 mM
glutathione	10 mM
hydroxylamine	100 mM
$CaCl_2$	5 mM
TGAse	5 mg/mL

Table seventeen: hydroxamate assay cuvette solution.

Ferric Chloride solution:

Equal volumes of
- 3 M HCl
- 5% $FeCl_3.6H_2O$ in 1 M HCl
- 12% CCl_3CO_2H

The solution prepared from the recipe in *table seventeen* was incubated at 37°C for five minutes. 0.75 mL of the above ferric chloride solution, was added and the precipitate was removed by low speed centrifugation. A UV spectrophotometer was calibrated against a blank containing the above solution with the TGAse omitted. The absorbance of the resulting solution was then read at

525 nm (*table eighteen*). The hydroxamate formed was proportional to enzyme concentration.

TGAse batch number	absorbance at 525 nm
1	0.525
2	0.311
3	0.073

Table eighteen: absorbance of hydroxamate assay solutions containing TGAse.

8.11.3 Investigation into the crosslinking reaction of high molecular weight glutenin subunits catalysed by transglutaminase[30]

Dried HMW glutenin subunits (15 mg) were added to a 0.1 M Tris-HCl (pH 6.8) solution containing 10 mM DTT (750 µL). The slurry was mixed vigorously to dissolve as much protein as possible. An aliquot was removed and stored at -10°C. A volume of a freshly prepared 1 mg/mL transglutaminase stock solution (95 µL) was then added and the solution was vortexed before being placed in an incubator at a temperature of 37°C, with continuous agitation. An aliquot was removed after thirty minutes, one, three, six and twenty four hours and stored at -10°C prior to analysis by SDS-PAGE.

8.11.4 Investigation into the crosslinking reaction of dough proteins catalysed by transglutaminase[31]

Two doughs were prepared by the 125 gram MDD method described below. The first dough contained only flour, water and salt whereas the second dough also contained 5000 p.p.m. transglutaminase. A small portion of each dough was frozen in liquid nitrogen, dried by lyophilisation before being ground into a flour with a mortar and pestle. The total proteins were then extracted from each flour by the following method.

The above flours (1.5 g) were suspended in 50% propan-1-ol (7.5 mL) at room temperature for one hour, with shaking every ten minutes. After high speed centrifugation (10000 g, 10 mins), the supernatant was removed and 50% propan-1-ol (5 mL) was added to the flour pellet which was then agitated at room temperature for thirty minutes. After a further extraction with 50% propan-1-ol (3 mL) for fifteen minutes, the supernatants were pooled and concentrated, to a volume of approximately 1 mL, by lyophilisation. A 30 μL was prepared for analysis by SDS-PAGE.

The soluble proteins were extracted from a further portion of the above flours (10 mg) by dissolution in a buffered solution (0.5% SDS, 0.05 M phosphate buffer, pH 6.9) (1 mL) for thirty minutes, at 20°C, with gentle agitation every ten minutes. The sample was centrifuged (10000 r.p.m.) for ten minutes. The insoluble proteins were extracted as above but were solubilised using a Branston sonifier, set at 15 W, for thirty seconds prior to centrifugation. SE-HPLC analysis of the supernatents from each extraction were carried out as described in section *8.8.2.* The % composition of the various protein components of the flours were calculated from the peak heights obtained by SE-HPLC.

8.12 *Experimental for work described in chapter six - The effect of transglutaminase on dough properties*

8.12.1 *General procedure for the preparation of 125 gram doughs*

Flour (125 g) was weighed into a metal bowl. The flour was warmed to approximately 20°C by resting the metal bowl in a tank of hot water, ensuring no water entered the bowl. Water (80 mL), with a temperature of approximately 10°C, was poured into a measuring cylinder to which salt (2.5 g) had been added. The flour was carefully added to the mixing bowl of a variable speed mixer. The salt solution was then added. Unless otherwise stated, the doughs were mixed at a constant speed of 150 r.p.m. The mixer was stopped once a work input of 9 Wh/kg had been attained, and the dough was removed.

The improvers ascorbic acid, potassium bromate and transglutaminase (*table nineteen*) were introduced to their respective doughs by suspending in the salt solution immediately prior to its addition to the flour.

improver	mass added (mg)	concentration (p.p.m.)
ascorbic acid	12.5	100
bromate	6.25	50
TGAse	125	1000
	200	1600
	312.5	2500
	625	5000
	1250	10000
	2500	20000

Table nineteen: concentrations and masses of improvers added to 125 g doughs.

Improver	Mean relaxation time (sec) at ten minute intervals after mixing								
	10	20	30	40	50	60	70	80	90
no improver	16.1	15.9	13.6	13.1	13.4	13.3	13.6	13.7	14.2
AA (100 p.p.m.)	28.2	27.6	27.7	28.4	29.9	27.5	30.0	30.8	31.2
AA/Bro (50 p.p.m. /100 p.p.m.)	27.3	31.5	28.2	29.1	27.9	30.4	32.0	32.1	32.6
TGAse (1000 p.p.m.)	17.3	14.9	15.8	19.5	21.8	22.8	29.1	33.4	37.6
TGAse (1600 p.p.m.)	22.3	20.4	22.0	27.2	28.7	30.8	34.8	44.3	49.5
TGAse (4000 p.p.m.)	18.6	19.0	20.1	22.3	28.3	28.2	33.6	40.8	44.7
TGAse (6000 p.p.m.)	19.4	20.1	31.6	40.9	41.0	61.3	80.3	82.0	102.6

Table twenty: effect of different flour improvers on the relaxation time of dough.

8.12.2 The effect of transglutaminase on dough relaxation times

Table twenty: effect of different improvers on the dough relaxation time sixty minutes after mixing.

Non-yeasted doughs, prepared from the above recipe, were rapidly weighed into 16 gram portions using metal scissors to cut the dough. The portions were then passed through a 2.5 mm sheeter which flattened and elongated the dough. The dough was then hand-moulded, between parallel plates, producing an oval roll of dough approximately 6 cm long and 2.5 cm in diameter.[32] The dough was enclosed in a metal pattie tin and rested in an oven at 32°C, with a humidity level of 85%, for 90 minutes. As soon as the first 16 gram portion had been moulded, the relaxation time of the dough was tested. This was the zero-time control dough. Further portions were tested at ten minute intervals for the duration of the experiment.

8.12.3 Effect of transglutaminase on the work input of a dough

Triplicate doughs containing the above concentrations of improver, were mixed on a Mitchell 1000 Electronic Dough Developer interfaced with a Sekonic SS 250F recorder. They were mixed to a constant work input of 15 Wh/kg and a water level of 60% flour weight. The peak work input requirement for that dough, correlated with the resulting mixing graph, was electronically recorded by the mixer (*table twenty one*).

The relaxation times of the doughs were measured using an Instron universal testing machine (UTM) (model 1011, interfaced to a personal computer). A crosshead speed of 20 mm/min, a load range of 1000 g and a sampling speed of 20 Hz were selected. The dough was removed from the prooving oven and, still resting on the metal lid, was positioned on the template of the Instron, directly below the metal probe. The probe was then moved down onto the dough, compressing it to a force of 1.5 N, at which point the probe was stopped. The time taken for the dough to relax by 1 N, as measured by the stationary probe, was the recorded

relaxation time. All measurements were taken in triplicate and an average of the three measurements were recorded (*table twenty*).

improver	concentration (p.p.m.)	mean work input (Wh/kg)	raw work input (Wh/kg)
no improver	-	10.0	9.6
			10.0
			10.3
ascorbic acid	100	11.7	11.6
			11.7
			11.8
ascorbic acid/ bromate	100/50	10.3	9.8
			10.4
			10.6
TGAse	1600	9.7	9.1
			9.4
			10.5
	2500	9.3	9.1
			9.4
			9.5
	5000	9.0	8.9
			9.0
			9.1

Table twenty one: effect of different improvers on the work input requirement of the dough.

8.13 Effect of transglutaminase on the properties of bread

8.13.1 Preparation of stock solutions for the 50 gram mini bake method

Each of the following solutions were freshly prepared immediately prior to each bake.

Solution A

salt	50.0 g
sugar	18.75 g

made up to 1 L with water

Solution B

salt	25.0 g
sugar	9.375 g
ascorbic acid	0.25 g

made up to 500 mL with water

Solution C

salt	25.0 g
sugar	9.375 g
potassium bromate	0.125 g

made up to 500 mL with water

Solution D

yeast	50.0 g

the yeast was suspended in 200 mL of water, any lumps were dispersed, and then made up to 2 L with water

8.13.2 General procedure for the preparation of 50 gram mini bake loaves[33]

Bread was prepared using the 50 gram MDD bake test method, according to the recipe below (*table twenty two*). WRIfat contained 20% sodium stearoyl-2-lactylate, 15% diacetyl tartaric esters of distilled monoglycerides, 20% enzyme activate soya flour was used and was made up to 100% with ordinary flour and sufficient anti-caking agent to maintain good flowing properties.

ingredient	standard loaf (no improver)	ascorbic acid (100 p.p.m.)	ascorbic acid/ bromate (100/50 p.p.m.)
flour	50 g	50 g	50 g
solution A	20 mL	-	-
solution B	-	20 mL	10 mL
solution C	-	-	10 mL
solution D	5 mL	5 mL	5 mL
water	7.5 mL	7.5 mL	7.5 mL
WRIfat	0.6 g	0.6 g	0.6 g

Table twenty two: recipe for the preparation of 50 gram doughs.

Dough was prepared according to the above recipe and was mixed to a constant work input of 15.4 Wh/kg. The flour and WRIfat were added to the mixing bowl of a variable speed Mitchell 1000 Electronic Dough Developer Each of the solutions were sequentially added to the dry ingredients. After mixing, the dough was removed and its temperature recorded. It was then mechanically moulded and enclosed in a metal pattie tin before placing in a prooving oven at 32°C for eight minutes. The dough was moulded using a mechanical moulder, placed in a rectangular baking tin, of dimensions 68 mm x 68 mm x 66 mm, and put in a second prooving oven set at 40°C, with a humidity of 80-90%, for forty five minutes. It was then baked at 210°C for twenty five minutes. The loaf was allowed to cool before storing in a sealed plastic bag, at room temperature, prior to physical analysis.

Transglutaminase was introduced to the dough as a suspension in water, prepared immediately prior to its addition to the flour.

8.13.3 General procedure for the preparation of 1 kilogram large bake loaves

Bread was prepared using the 1 kilogram MDD bake test method, according to the recipe below (*table twenty three*).

ingredient	quantity
flour	1 kg
water	640 mL
yeast	30 g
salt	20 g
sugar	7.5 g
WRIfat	1.5 g

Table twenty three: recipe for the preparation of 1 kilogram doughs.

The dried ingredients were added to the mixing bowl and were mixed at 86 Wh/kg as the water was slowly poured in. Once the water had begun to mix with the dry ingredients, the mixing speed was increased to 328 r.p.m. and the dough was mixed to 82.5 counts (15 Wh/kg), as judged by a watt hour meter. After mixing, the dough was removed and its temperature recorded. It was prooved under plastic lids for ten minutes before moulding with a mechanical moulder. The dough was split into an 830 g portion and a 794 g portion. The 830 g dough was divided into roughly four equal parts which were individually moulded using a Mono Universal moulder, with a pressure board setting of 11.5 and a sheeting roll setting of 2. The pieces were laid across a rectangular baking tin of dimensions 24.4 cm x 13.5 cm x 11 cm. A lid was attached and the dough was prooved at 40°C and with a humidity of 80% for a further fifty minutes. The tin was then transferred to an oven, with both upper and lower heating, at a temperature of 210°C for twenty five minutes. The 794 g dough was moulded as a single piece using a Mono Universal moulder with a pressure board setting of 22 and a sheeting roll setting of 3. After moulding, it was placed lengthwise in a rectangular baking tin of dimensions 21.3 cm x 11.8 cm x 11 cm. The dough was prooved at 40°C for a further fifty minutes before transferring to an oven, with lower heating only, at a temperature of 235°C. After baking the loaves were allowed to cool and were stored in sealed plastic bags at room temperature prior to physical analysis.

Improvers were suspended in water prior to addition to the dry ingredients.

8.13.4 Effect of transglutaminase on bread volume

50 gram loaves of bread		
improver	concentration (p.p.m.)	volume (mL)
standard	-	285
		280
ascorbic acid	100	315
		315
ascorbic acid/ bromate	100/50	345
		345
TGAse	1000	260
		285
	1600	270
		305
	2500	295
		295
	5000	295
		300

One day after baking, the volume of the 50 gram loaves and the 1 kilogram loaves were measured. A large rectangular tin was placed in a metal basin. The tin was completely filled with rape seeds, the top levelled and the excess seeds were removed. The seeds remaining in the tin were then poured into a measuring cylinder and their volume recorded. An unsliced loaf of bread was then placed inside the tin and the tin again filled with rape seeds. The top was levelled and the volume recorded as before. The volume was calculated by subtracting this value from the volume of the tin containing only rape seeds (*table twenty four*).

1 kilogram loaves of bread		
improver	concentration (p.p.m.)	volume (L)
ascorbic acid/ bromate	100/50	3.31
TGAse	2500	3.29
	5000	3.39
	10000	2.69

Table twenty four: the effect of TGAse on the volume of 50 gram and 1 kilogram loaves of bread.

8.13.5 Effect of transglutaminase on bread texture[34]

Texture analysis was carried out one day after baking. The loaf of bread was sliced using a Mono reciprocating bread slicer, with a slice thickness of 12.5 mm, ensuring that the slices were flat and the two sides were parallel. Two slices from each loaf, equidistant from each other and the ends of the loaf, were selected. Images of the slices were captured using VideoPro 32 Image Analysis System. Each slice was placed on a matt black card and lit with a 150 W tungsten lamp from each end at a low angle. The camera was a Panasonic CL-700 colour 0.5 inch CCD surveillance camera fitted with a Cosmicar 66.67 inch, 25 mm TV lens. The slice was positioned beneath the camera so that it filled the screen without any of the crust of the slice showing.

The computer then evaluated the texture of the bread slice by calculating the cumulative pore sizes of that slice (*table twenty five*).

improver	concentration (p.p.m.)	raw texture score	
		lidded	unlidded
ascorbic acid/ bromate	100/50	9.51	9.78
		9.88	9.98
		10.78	10.06
		9.78	11.27
TGAse	2500	9.12	9.24
		9.70	10.65
		10.01	11.70
		12.31	12.45
	5000	9.53	8.36
		10.23	9.94
		10.26	10.47
		10.75	11.66
	10000	7.65	8.41
		8.12	9.62
		8.24	9.91
		8.74	11.45

Table twenty five: effect of different improvers on the texture of 1 kilogram loaves of bread.

8.13.6 Effect of transglutaminase on crumb strength

One day after baking, loaves were machine sliced to a thickness of 12.5 mm. Crumb strengths of individual slices were measured using an Instron UTM (model 1011), using a modification of the methods of Dahle and Montgomery[35] and Morgenstern *et al.*,[36] the latter was developed for measuring the extensional properties of dough sheets.

50 gram loaves of bread			
improver	concentration (p.p.m.)	mean crumb strength	raw crumb strength data
no improver	-	2.03	1.98
			2.05
			2.05
ascorbic acid	100	2.20	2.15
			2.20
			2.24
ascorbic acid/ bromate	100/50	2.53	2.34
			2.60
			2.64
TGAse	1000	2.63	2.52
			2.58
			2.78
	1600	2.85	2.80
			2.81
			2.93
	2500	3.21	3.08
			3.19
			3.35
	5000	4.04	3.97
			4.02
			4.14
	10000	6.46	6.13
			6.37
			6.88

1 kilogram loaves of bread			
improver	concentration (p.p.m.)	raw crumb strength data	
		lidded	unlidded
ascorbic acid/ bromate	100/50	3.74	2.63
		3.78	2.64
		3.87	2.69
TGAse	2500	4.10	3.07
		4.34	3.25
		4.76	3.42
	5000	4.70	3.43
		4.72	3.47
		4.91	3.56
	10000	7.24	4.74
		8.85	4.88
		8.91	5.43

Table twenty six: effect of different improvers on the crumb strength of bread prepared using the MDD bake method.

A central slice of bread was placed between two perspex plates which had a circular aperture in the centre of each plate. The deforming device consisted of two Perspex plates, each with a 42 mm circular aperture in the centre. The slice was held by eight sharp pins that were set in a circle 20 mm from the apertures of both plates. The pins were 1.2 mm in diameter with sharpened ends, and protruded approximately 2 mm into the bread slice. A 15 mm probe, with a flat end, was attached to the Instron. The Perspex plates, containing the slice of bread, were positioned so that the centre of the aperture was directly below the probe. The probe then moved vertically down through the centre of the aperture with a crosshead speed of 100 mm/min. The maximum force at rupture, the crumb strength, was recorded (*table twenty six*).

8.14 References

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X-RAY CRYSTALLOGRAPHIC DATA FOR CYCLOTENE HYDRATE

Table 1 lists the crystallographic data for cyclotene hydrate. Data were collected with a Siemens SMART CCD area detector on a colourless block measuring 0.54 x 0.46 x 0.39 mm, using graphite monochromatised Mo Kα radiation (λ = 0.71073 Å). A total of 3259 reflections with 5.72° < 2θ < 52.88° were collected. The structure was solved by direct methods and refined (all non-hydrogen atoms anisotropic) on F^2, using all 1258 independent data. C-H hydrogen atoms were included in calculated positions, while all hydrogen atoms attached to oxygen were located from difference Fourier calculations and refined isotropically.

Table 2 lists the final atoms coordinates and equivalent isotropic displacement parameters. Final bond lengths and angles are listed in Table 3.

Table 1. Crystallographic Data for Cyclotene Hydrate.

$C_6H_8O_2.H_2O$	space group P-1
fw = 130.14 g mol^{-1}	Z = 2
a = 6.8829(2) Å	D_x = 1.316 g cm^{-3}
b = 6.9979(3) Å	λ = 0.71073 Å
c = 7.8655(3) Å	μ = 0.105 mm^{-1}
α = 111.529(1) °	T = -120 °C
β = 95.615(2) °	F(000) = 140
γ = 106.786(2)°	R[a] = 0.0445
V = 328.45(2) Å^3	wR[b] = 0.1156 (all 1258 data)

[a] $R = \Sigma||F_o| - |F_c||/\Sigma|F_o|$ for 2840 data with $I > 2\sigma(I)$.

[b] $wR = (\Sigma[w(F_o^2 - F_c^2)2]/\Sigma[w(F_o^2)2])^{1/2}$.

Table 2. Final atomic coordinates for Cyclotene Hydrate.

atom	x	y	z	U_{eq}
O1	0.3429(2)	0.7178(2)	1.28819(15)	0.0322(3)
O2	0.3398(2)	0.7767(2)	0.9591(2)	0.0297(3)
C1	0.2764(2)	0.5590(3)	1.1355(2)	0.0248(4)
C2	0.2698(2)	0.5736(2)	0.9553(2)	0.0226(3)
C3	0.1879(2)	0.3735(3)	0.8132(2)	0.0255(4)
C4	0.1304(3)	0.1988(3)	0.8854(2)	0.0319(4)
C5	0.1879(2)	0.3217(3)	1.0997(2)	0.0296(4)
C6	0.1513(3)	0.3193(3)	0.6083(2)	0.0345(4)
H21	0.3605(32)	0.7736(34)	0.8516(32)	0.044
H4A	0.2094(3)	0.0986(3)	0.8438(2)	0.038
H4B	-0.0205(3)	0.1120(3)	0.8399(2)	0.038
H5A	0.0631(2)	0.2927(3)	1.1522(2)	0.036
H5B	0.2924(2)	0.2773(3)	1.1562(2)	0.036
H6A	0.0026(4)	0.2391(19)	0.5501(4)	0.052
H6B	0.2316(16)	0.2282(18)	0.5503(4)	0.052
H6C	0.1956(19)	0.4552(3)	0.5898(2)	0.052
O3	0.4527(2)	0.8392(2)	0.6698(2)	0.0380(4)
H31	0.5197(38)	0.9801(43)	0.7027(33)	0.057
H32	0.4060(37)	0.7830(39)	0.5502(38)	0.057

Table 3. Final bond distances and angles for Cyclotene Hydrate.

O1 - C1	1.227(2)	O2 - C2	1.350(2)
O2 - H21	0.86(2)	C1 - C2	1.456(2)
C1 - C5	1.499(2)	C2 - C3	1.342(2)
C3 - C6	1.491(2)	C3 - C4	1.499(2)
C4 - C5	1.531(2)	O3 - H31	0.88(3)
O3 - H32	0.86(3)		
C2 - O2 - H21	112.0(14)	O1 - C1 - C2	124.30(14)
O1 - C1 - C5	127.38(14)	C2 - C1 - C5	108.32(13)
C3 - C2 - O2	132.23(14)	C3 - C2 - C1	110.82(14)
O2 - C2 - C1	116.94(13)	C2 - C3 - C6	127.21(15)
C2 - C3 - C4	110.99(14)	C6 - C3 - C4	121.80(13)
C3 - C4 - C5	105.34(13)	C1 - C5 - C4	104.53(13)
H31 - O3 - H32	105.9(22)		

ELECTROSPRAY MASS SPECTROMETRY DATA FOR THE CYCLOTENE-RIBONUCLEASE A REACTION PRODUCTS

Description: SEF 4/74/1 control, cone +45, MeCN/H2O

Component: A

Rel.Intensity	m/z	Charges	Molecular Mass	
2.84	651.86	21	13667.86	
3.53	685.00	20	13679.86	
8.84	720.31	19	13666.78	Rejected
5.42	761.61	18	13690.87	
6.27	805.77	17	13680.93	
8.72	856.15	16	13682.30	
8.18	913.61	15	13688.97	
7.52	978.67	14	13687.22	
6.18	1052.92	13	13674.88	
7.45	1141.28	12	13683.27	
11.21	1244.70	11	13680.61	
29.24	1368.95	10	13679.45	
55.31	1520.98	9	13679.78	
100.00	1710.88	8	13678.98	
84.25	1955.09	7	13678.58	
27.41	2281.24	6	13681.41	
363.52		Mean:	13681.00	+/- 5.37 SD

Description: SEF 4/64/1 day 4, cone +45, MeCN/H2O

Component: A

Rel.Intensity	m/z	Charges	Molecular Mass	
26.85	856.20	16	13683.02	
24.90	913.57	15	13688.43	
21.88	978.16	14	13680.17	
21.38	1052.58	13	13670.42	Rejected
24.29	1141.43	12	13685.09	
58.87	1244.75	11	13681.13	
86.87	1368.95	10	13679.39	
94.45	1520.86	9	13678.64	
68.39	1711.47	8	13683.66	
30.74	1955.65	7	13682.47	
14.50	2280.09	6	13674.51	
451.75		Mean:	13681.65 +/- 3.63 SD	

Component: B

Rel.Intensity	m/z	Charges	Molecular Mass
19.99	861.63	16	13769.98
25.96	919.95	15	13784.14
23.22	984.71	14	13771.86
20.46	1061.96	13	13792.38
21.53	1149.06	12	13776.59
43.86	1254.20	11	13785.14
72.55	1379.38	10	13783.74
96.34	1532.41	9	13782.63
100.00	1723.98	8	13783.75
57.89	1969.57	7	13779.95
20.83	2297.71	6	13780.23
502.65		Mean:	13780.95 +/- 6.03 SD

Description: SEF 4/64/1 day 4, cone +45, MeCN/H2O

Component: C

Rel.Intensity	m/z	Charges	Molecular Mass	
5.81	514.74	27	13870.74	
8.55	535.68	26	13901.35	
7.75	555.34	25	13858.27	Rejected
6.28	578.80	24	13867.01	
8.55	605.29	23	13898.46	
9.46	632.39	22	13890.39	
11.30	661.90	21	13878.64	
13.61	695.77	20	13895.15	
25.22	731.51	19	13879.47	
17.62	772.57	18	13888.14	
22.76	817.41	17	13878.92	
26.03	869.41	16	13894.45	
23.46	925.49	15	13867.22	
21.66	992.95	14	13887.26	
18.29	1067.90	13	13869.54	
20.78	1158.62	12	13891.35	
25.37	1263.67	11	13889.33	
45.58	1389.97	10	13889.67	
64.13	1543.45	9	13881.94	
73.83	1736.57	8	13884.50	
60.63	1983.90	7	13880.23	
25.06	2314.04	6	13878.22	
533.99		Mean:	13883.90 +/- 9.78 SD	

Description: SEF 4/64/1 day 4, cone +45, MeCN/H2O

Component: D

Rel.Intensity	m/z	Charges	Molecular Mass	
5.42	501.50	28	14013.65	Rejected
6.67	519.26	27	13992.79	
7.79	539.82	26	14009.18	Rejected
7.49	561.31	25	14007.49	
8.85	583.54	24	13980.66	
10.77	608.37	23	13969.31	
8.58	636.71	22	13985.51	
16.76	666.86	21	13982.92	
16.95	699.83	20	13976.35	
19.32	736.81	19	13980.24	
20.87	778.08	18	13987.22	
22.92	823.80	17	13987.52	
26.38	874.91	16	13982.37	
21.67	933.06	15	13980.82	
22.04	1000.56	14	13993.75	
17.40	1076.38	13	13979.80	
16.42	1166.09	12	13980.98	
16.34	1272.83	11	13990.09	
31.88	1398.91	10	13979.05	
42.74	1555.11	9	13986.91	
56.19	1748.40	8	13979.15	
46.82	1998.21	7	13980.44	
23.01	2331.02	6	13980.10	
460.07		Mean:	13983.97 +/- 7.59 SD	

Description: SEF 4/64/1 day 4, cone +45, MeCN/H2O

Component: E

Rel.Intensity	m/z	Charges	Molecular Mass	
6.99	505.24	28	14118.39	
9.49	523.30	27	14101.91	
6.46	543.07	26	14093.73	
8.40	564.16	25	14078.78	
7.63	588.88	24	14108.85	
9.35	613.34	23	14083.58	
11.31	640.83	22	14076.07	
12.49	671.28	21	14075.82	
12.42	704.58	20	14071.35	
16.67	742.72	19	14092.59	
18.77	783.84	18	14091.03	
25.30	830.04	17	14093.55	
25.59	882.23	16	14099.48	
29.82	941.01	15	14099.97	
21.11	1007.37	14	14089.12	
18.34	1084.59	13	14086.61	
16.10	1174.61	12	14083.19	
14.63	1281.00	11	14079.90	
18.72	1410.23	10	14092.23	
28.59	1566.58	9	14090.18	
32.25	1768.01	8	14136.04	Rejected
28.83	2019.61	7	14130.22	
15.97	2357.65	6	14139.87	Rejected
347.01		Mean:	14092.22 +/- 14.00 SD	

Thus grew the tale of Wonderland:
Thus slowly, one by one,
Its quaint events were hammered out -
And now the tale is done,
And home we steer, a merry crew,
Beneath the setting sun.

Lewis Carroll
"Alice's Adventures in Wonderland"